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PRESYNAPTIC INHIBITORY ACTION OF 2-SUBSTITUTED ADENOSINE DERIVATIVES IN RAT VAS DEFERENS: ROLE OF ADENOSINE UPTAKE AND DEAMINATION

by

(C) MILAN J. MULLER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Presynaptic inhibitory action of 2-substituted adenosine derivatives in rat vas deferens: Role of adenosine uptake and deamination", submitted by Milan J. Muller in partial fulfilment of the requirements for the degree of Master of Science.

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Supervisor

.....

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Date: Nov. 16/78

For my family; Valeria, Milan, Mary and Andy.

ABSTRACT

Adenosine and adenine nucleotides have been shown to inhibit noradrenergic transmission in various tissues including the rat vas deferens (Clanachan et al., 1977). Structural requirements for adenosine activity appear to be similar for pre- and postsynaptic receptors in smooth muscle (cf. Paton et al., 1978). The present study compares the presynaptic effects of 2-substituted adenosine derivatives with those of adenosine and deals with possible reasons for differences of potency and duration of action.

Adenosine and 2-substituted adenosine derivatives inhibited submaximal twitch and sustained responses of rat vas deferens to electrical field stimulation significantly more than submaximal responses to exogenous noradrenaline, suggesting a predominately presynaptic site of action. Further, since inhibition by adenosine derivatives was antagonized by theophylline but not by phenoxybenzamine, atropine or indomethacin, these compounds probably act via specific receptors.

The order of inhibitory potency was as follows: 2-Chloroadenosine $\geq$ 2-hydroxyadenosine $\geq$ 2-bromoadenosine $\geq$ 2-fluoroadenosine > 2-aminoadenosine = adenosine. Further, the more potent derivatives were also longer acting than adenosine. A comparison of our structure-activity data on presynaptic action of adenosine and derivatives with those on postsynaptic direct smooth muscle stimulation in the literature shows much similarity.

HNBTG or dipyridamole and 2'-deoxycoformycin, inhibitors of adenosine uptake and deamination respectively, potentiated the inhibitory activity of adenosine on nerve-mediated responses of rat vas deferens. In contrast, inhibition by 2-chloroadenosine was unaltered by these

agents. Similar results were obtained with other 2-substituted adenosine derivatives. These results suggest that the inhibitory activity of adenosine is masked by both uptake and deamination in this tissue. Further, experiments involving inhibition of both uptake and deamination of adenosine indicated that adenosine and 2-chloroadenosine were equipotent. However, the long duration of action of 2-chloroadenosine still is not explained by these processes. Therefore, caution is required in interpreting the relative potencies and dose-response curves of adenosine and adenosine derivatives which are subject to uptake and deamination.

Inhibitors of adenosine uptake and deamination did not significantly alter frequency-response determinations of rat vas deferens, thus adenosine may not normally modulate noradrenergic transmission in this tissue. The possibility that adenosine is of functional importance in this respect at low frequencies of stimulation or under anoxic conditions has not been examined. The observation that inhibitors of adenosine uptake and deamination facilitated antagonism by theophylline of adenosine action warrants further investigation.

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INTRODUCTION

GENERAL

The noradrenergic nerve terminal is the site of noradrenaline synthesis, storage, release, uptake and inactivation. Transmitter release from the nerves is regulated by the flow of electrical pulses, which themselves are controlled by the action of various agents on somatic-dentritic sites. This classical understanding has recently been expanded by the realization that noradrenaline release can also be modulated by endogenous factors interacting with receptors presumably located on the extracellular surface of noradrenergic nerve terminals. These factors include noradrenaline, dopamine, acetylcholine, prostaglandins, angiotensin and enkephalins. More recently, adenosine and the adenine nucleotides (ATP, ADP and AMP) have also been considered to play such a role.

In the course of investigating various aspects of presynaptic action of adenosine modulating transmitter release, structure-activity requirements for presynaptic inhibition by adenosine have been studied in the rat vas deferens by Paton et al. (1978). In many other adenosine sensitive systems, 2-substituted adenosine derivatives have been shown to be more potent and longer acting than the parent compound, without adequate explanation for these differences. We have therefore expanded structure-activity studies with the aim of understanding why 2-substitution of adenosine should result in a more potent compound. In the introduction of this dissertation, the presynaptic inhibition by adenosine of noradrenergic transmission and processes which may limit the potency of adenosine (i.e., uptake and metabolism) are reviewed and discussed.

PRESYNAPTIC MODULATION OF NORADRENERGIC TRANSMISSION

Brown and Gillespie (1957) observed that nerve stimulated overflow of noradrenaline from the perfused cat spleen was increased by phenoxybenzamine, an alpha-adrenoceptor antagonist, and explained this finding by hypothesising that the alpha-adrenoceptor, located postsynaptically, was an important site of loss of noradrenaline. It is now clear that, in fact, this result was based on presynaptic feedback. Attempts by many investigators to explain this finding eventually lead to the discovery of presynaptic alpha-adrenoceptors.

It is now generally accepted that noradrenaline has a feedback action on alpha-adrenoceptors located on the noradrenergic nerve terminal to inhibit its own release. Phenoxybenzamine then increases noradrenaline release by inhibiting this feedback mechanism (cf. Langer, 1977; Starke, 1977; Westfall, 1977).

Besides the noradrenaline-mediated feedback system, a number of other presynaptic receptor systems have been found. In various systems, the following agents facilitate noradrenaline release induced by nerve stimulation: beta-Adrenergic agents, nicotine, prostaglandins ($PGF_{2\alpha}$) and angiotensin. On the other hand, stimulation of other presynaptic receptors by the following agents inhibits noradrenaline release induced by nerve stimulation: alpha-Adrenergic agents, dopamine, muscarinic drugs, prostaglandin (PGE_2), enkephaline and adenosine. For comprehensive reviews of presynaptic modulation of noradrenergic transmission see Starke (1977) or Westfall (1977).

ATP AND ITS METABOLITES

Since ATP is a major intracellular energy source in mammals, it is

found ubiquitously and in abundant amounts. Apart from this function, ATP and its metabolites (ADP, AMP and adenosine) have been shown to have variable actions in many biological systems (cf. Burnstock, 1972). Physiologically, it has been suggested that purine nucleotides and/or adenosine may regulate blood flow in the heart (Berne, 1963), skeletal muscle (Boyd and Forrester, 1968), and brain (Rubio et al., 1975). ATP may also release active amines from mast cells (cf. Kiernan, 1972) and from adrenal chromaffin granules (Smith and Winkler, 1972). Further, the possibility of a third component in the autonomic nervous system, which releases ATP or related compound as its neurotransmitter (Burnstock et al., 1970) has been reviewed (cf. Burnstock, 1972, 1975). These nerves were termed "purinergic" nerves by Burnstock (1971). This hypothesis of ATP as a direct neurotransmitter substance remains to be proven. However, the presence of ATP in various nerve granules and its co-release upon stimulation of adrenergic nerves or cholinergic nerves raises the question of a physiological role of ATP in this connection, possibly as a co-transmitter or modulator. The term purinergic will be used in this dissertation to generally refer to adenosine receptors or adenosine-related functions.

INHIBITION OF TRANSMITTER RELEASE BY ADENOSINE

Adenosine and the adenine nucleotides have been shown to inhibit sympathetic neurotransmission in numerous tissues, both in vivo and in vitro. In vivo studies have demonstrated a presynaptic inhibitory action on noradrenaline release in rabbit kidney, canine subcutaneous adipose tissue (Hedqvist and Fredholm, 1976; Fredholm, 1974) and saphenous vein (Verhaeghe et al., 1977). In vitro studies have yielded

similar results in canine (Verhaeghe et al., 1977) and rabbit (Su, 1978) saphenous vein, rat (Enero and Saidman, 1977) and rabbit (Su, 1974, 1978), portal vein, canine tibial artery (Verhaeghe et al., 1977), rabbit pulmonary and ear arteries (Su, 1978), rabbit kidney and guinea-pig vas deferens (Hedqvist and Fredholm, 1976) and rat vas deferens (Clanachan et al., 1977).

Inhibition of transmitter release by adenosine and the adenine nucleotides has also been shown at parasympathetic nerve terminals (Sawynok and Jhamandas, 1976; Vizi and Knoll, 1976; Leighton and Parmeter, 1977), skeletal motor nerve terminals (Ginsborg and Hirst, 1972; Ribeiro and Walker, 1975) and perhaps autonomic ganglia (Theobald and de Groat, 1977). Furthermore, adenosine is released in the brain and modifies central neuronal activity (Sulakhe and Phillis, 1976).

Su (1978) has suggested that such "purinergic" feedback regulation may occur at all neuroeffector synapses. In this dissertation, discussion will be limited to the presynaptic inhibitory action of adenosine and the adenine nucleotides on noradrenergic transmission in the peripheral nervous system.

EVIDENCE FOR A PRESYNAPTIC SITE OF ACTION OF ADENOSINE

A presynaptic site of action of adenosine and the adenine nucleotides has been demonstrated both indirectly and directly in a number of noradrenergically innervated tissues. The evidence includes the demonstration that adenosine and the adenine nucleotides inhibit responses due to electrical nerve stimulation while the responses to exogenous noradrenaline are either not affected, as in the rat vas deferens (Clanachan et al., 1977), or much less inhibited, as in rabbit and

canine blood vessels (Su, 1978; Verhaeghe et al., 1976). Further, more direct evidence for a presynaptic site of action of adenosine and the adenine nucleotides results from the observation that adenosine and ATP decrease the amount of [^{3}H]-noradrenaline released by nerve stimulation in a number of tissues (Clanachan et al., 1977; Enero and Saidman, 1977; Hedqvist and Fredholm, 1976; Su, 1978; Verhaeghe et al., 1977).

EVIDENCE FOR THE PRESYNAPTIC LOCATION OF ADENOSINE RECEPTORS LOCATED EXTRACELLULARLY

Theophylline, a commonly used adenosine antagonist (cf. Burnstock, 1975), appears to competitively antagonize the direct postsynaptic actions of adenosine (Bünger et al., 1975; Osswald, 1975; Ally and Nakatsu, 1976). Furthermore, the presynaptic inhibition of acetylcholine release by adenosine has also been shown to be competitively inhibited by theophylline (Sawynok and Jhamandas, 1976; Vizi and Knoll, 1976; Okwuasaba et al., 1977). In addition, the presynaptic inhibitory actions of adenosine on noradrenergic transmission have been shown to be antagonized by theophylline (Clanachan et al., 1977; Verhaeghe et al., 1977; Su, 1978). This suggests that a similar purinergic receptor system is present in these tissues.

In some systems, adenosine and the adenine nucleotides seem to act via a common receptor. These compounds are equipotent presynaptic inhibitors in the rat vas deferens and rat portal vein (Clanachan et al., 1977; Enero and Saidman, 1977), while ATP appears to be slightly more potent than adenosine in the rabbit portal vein (Su, 1978). In the rat vas deferens the presynaptic action of both adenosine and adenine nucleotides were potentiated (Clanachan et al., 1977) by the adenosine

uptake inhibitors 2-amino-6-[(2-hydroxy-5-nitro)benzylthio]-9-β-D-ribofuranosylpurine (HNBTG) and dipyridamole (Paterson et al., 1975; Stafford, 1966). Since the adenine nucleotides do not readily traverse cell membranes (Hattori et al., 1969), these compounds may first be hydrolysed by extracellular phosphorylytic enzymes to form adenosine which then inhibits noradrenergic transmission (Clanachan et al., 1977).

Potentiation of the presynaptic inhibitory action of adenosine with inhibitors of its uptake also suggested at least in the rat vas deferens, that extracellular receptors were studied (Clanachan et al., 1977). More convincing evidence has been found to suggest the existence of a cell surface adenosine receptor on canine and guinea-pig myocytes (Olsson et al., 1976; Schrader et al., 1977) as well as on atrial muscle cells (Schrader et al., 1977). Olsson et al. (1976) have covalently linked adenosine via N_6 of the purine ring to periodate-oxidized stachyose which has a molecular weight of about 1000 daltons. Schrader et al., (1977) have coupled AMP to carbonic anhydrase forming a protein-AMP conjugate with a molecular weight of 30,000 daltons. The experimentors suggested that, since these adenosine derivatives are too large to enter cells but still show agonist activity, the adenosine receptor must be located extracellularly.

Evidence has accumulated, using receptor blocking drugs, to suggest that the inhibitory action of adenosine and the adenine nucleotides involves a specific receptor. These compounds appear not to stimulate beta-adrenoceptors, dopaminergic receptors (Su, 1978) or muscarinic receptors (Clanachan et al., 1977; Su, 1978). In the rat (Clanachan et al., 1977) and guinea-pig vas deferens and in rabbit kidney (Hedqvist and Fredholm, 1976) phenoxybenzamine did not alter the inhibition of

noradrenaline release by adenosine; however, the inhibitory effect of ATP was increased by phenoxybenzamine in the rat portal vein (Enero and Saidman, 1977). Therefore, although there appears to be no interaction between adenosine and alpha-adrenoceptors in some systems, in the rat portal vein this does not seem to be the case.

Although ATP and ADP are potent stimulators of the synthesis of prostaglandins (Needleman et al., 1974), which also inhibit noradrenergic transmission (Hedqvist, 1974), the adenine nucleotides probably do not act by prostaglandin synthesis (Hedqvist and Fredholm, 1976; Clanachan et al., 1977). However, limited synthesis of prostaglandins by ATP may occur in rabbit blood vessels (Su, 1978).

MECHANISM OF ACTION

Little is known concerning the mechanism of action by which adenosine and the adenine nucleotides inhibit noradrenergic transmission. Based on histofluorescence studies in the rat retina, Angelakos (1964) has suggested that ATP may enhance noradrenaline uptake. However, Su (1978) has found no supportive evidence for this in the rabbit portal vein. Furthermore, this author demonstrated that in the presence of desipramine and hydrocortisone, i.e., inhibitors of noradrenaline uptake, the neurogenic constriction of the ear artery was still inhibited by ATP. In addition, ATP did not alter either the initial rate or net accumulation of [^{3}H]-noradrenaline in the portal vein (Su, 1978) or increase the level of metabolites in blood vessels (Verhaeghe et al., 1977). Therefore, ATP and adenosine probably do not alter noradrenaline uptake or metabolism. However, high adenosine concentrations may increase the intra-neuronal leakage of noradrenaline from storage

vesicles (Verhaeghe et al., 1977).

Adenosine has been shown to effectively increase cAMP content in brain slices (Mah and Daly, 1976), heart slices (Huang and Drummond, 1976) and in the rabbit vagus nerve (Roch and Salamin, 1976). However, cAMP may facilitate rather than inhibit noradrenergic transmission in the perfused cat spleen (Cubeddu et al., 1975) and differences have been found between structural requirements necessary for presynaptic adenosine effects and its interaction with adenylate cyclase (Paton et al., 1978). Thus, Baer and Paton (1978) have concluded that available evidence suggests that cAMP is not involved in the presynaptic inhibitory action of adenosine, although this question has not been investigated directly.

While the mechanism of action of the presynaptic inhibition of noradrenergic transmission by adenosine and the adenine nucleotides remains obscure, it is likely that control of calcium fluxes or binding is involved. Adenosine (Verhaeghe et al., 1977) and ATP (Su, 1978) inhibited [^{3}H]-noradrenaline release due to nerve stimulation in blood vessels and reduced the release of [^{3}H]-noradrenaline due to high K^+. Conversely, tyramine-induced release of [^{3}H]-noradrenaline was not altered. Both nerve stimulation and high K^+-induced release have an absolute requirement for external calcium (Kirpekar and Wakade, 1968) while release by tyramine, an indirectly acting sympathomimetic, occurs in the absence of extracellular calcium (Thoenen et al., 1969; Westfall and Brasted, 1972). Furthermore, the demonstration that adenosine and the adenine nucleotides have a greater inhibitory effect at low frequencies of stimulation than at higher frequencies (Verhaeghe et al., 1977; Su, 1978) is consistent with the concept that these compounds act

by limiting the amount of available calcium. Presumably, at higher frequencies of stimulation enough calcium is available for secretion to overcome the inhibition by adenosine. More support for this hypothesis would come from experiments in which the external calcium concentration were either raised or lowered. In this respect, it has been shown that noradrenaline is more effective at inhibiting its own release when the external calcium concentration is low (cf. Starke, 1977).

Thus, the exact mechanism of action of the presynaptic inhibition by adenosine is not known, but it appears that the mechanism is probably similar to that of the other presynaptic inhibitors and involves modulation of cellular calcium influx or efflux. Alternatively, depolarization or hyperpolarization of the neuronal membrane could explain facilitation or inhibition of noradrenaline release. It is also possible that the presynaptic modulators may interfere with a further step in the excitation-secretion coupling process (cf. Starke, 1977; Westfall, 1977).

SOURCES OF PURINERGIC MODULATOR(S)

The pharmacological effects of purines in many systems are undisputable; however, their physiological regulatory functions remain to be established and proven. An endogenous source of purines would be required if a physiological role exists, and it is of interest to discuss this possibility. Four possible sources of the purine modulator(s) of noradrenergic transmission are enumerated and discussed below:

(1) Purinergic nerves;

(2) Noradrenergic nerves;

(3) Cholinergic nerves;

(4) Postsynaptic neuroeffector tissues.

Stimulation of purinergic fibres in the vagus nerves to the stomach increases the venous efflux of adenosine and inosine, breakdown products of ATP, in both guinea-pigs and toads (Burnstock et al., 1970; Satchell and Burnstock, 1971). More direct evidence of the release of ATP from purinergic nerves in the taenia coli have resulted using the highly sensitive luciferin-luciferase firefly assay technique (cf. Burnstock, 1975).

ATP is released together with catecholamines from adrenal medullary vesicles and whole glands (Douglas, 1968; Stevens et al., 1972), and it seems possible that some ATP is released with noradrenaline from noradrenergic nerves (Geffen and Livette, 1971; Su et al., 1971) since ATP is present in the noradrenaline storage vesicle. Langer and Pinto (1976) have suggested that ATP may be released with noradrenaline from noradrenergic nerves in the cat nictitating membrane.

Synaptic vesicles of cholinergic nerves in the electric organ of torpedine rays have an ATP to acetylcholine ratio of about 1 : 11 (Whittaker et al., 1972; Bohan et al., 1973). However, there is no evidence that ATP is released in association with acetylcholine from autonomic nerves (cf. Burnstock et al., 1978). It has been suggested that ATP is released with acetylcholine from phrenic nerves supplying the rat diaphragm (Silinsky and Hubbard, 1973). However, Zimmerman and Whittaker (1974) are concerned that the fall in vesicular ATP by nerve stimulation may be simply due to hydrolysis. Recently, coenzyme A, which contains the adenosine moiety in its structure, also has been considered as a possible modulator of acetylcholine output (Cook et al., 1978).

It is probable that ATP and metabolite(s) may be released in

response to hypoxia and exercise, not necessarily from neuronal sites, into the circulation of the brain, skeletal muscle and myocardium (Berne et al., 1974; Bockman et al., 1976; Paddle and Burnstock, 1974). Therefore, a postsynaptic site of release of purinergic modulator(s) is also possible.

PHYSIOLOGICAL SIGNIFICANCE

Purinergic modulation of transmitter release may be widespread (Su, 1978). As discussed above, ATP may be stored and released from a variety of neuronal and extraneuronal sites. A physiological role for purinergic modulation of noradrenergic transmission would, however, require sufficiently high concentrations of ATP or metabolites to reach the presynaptic receptor. Although the concentration of active compounds around the receptor cannot yet be measured, Su (1978) has suggested that sufficient concentrations may be achieved in many blood vessels. Based on pharmacological data, it appears that adenosine concentrations above 10 uM are often required for presynaptic inhibition of noradrenergic transmission.

Inhibitors of adenosine uptake and deamination, such as dilazep and erythro-9-(2-hydroxy-3-nonyl)adenine respectively, reduced the mechanical activity and the nerve stimulation-induced overflow of [^{3}H]-noradrenaline from the rat portal vein (Enero and Saidman, 1977). It was suggested that this was due to the raising of the effective concentration of adenosine released from the nerve. However, a critical experiment that was not carried out was to investigate whether a purinergic receptor blocker would reverse this effect of inhibiting adenosine uptake and deamination. Furthermore, if purinergic transmitter

modulation were of physiological significance one would expect that theophylline alone would increase noradrenaline and dopamine-β-hydroxylase release. This has been demonstrated with alpha-adrenoceptor antagonists (cf. Starke, 1977; Westfall, 1977), but not as yet with a purinergic receptor antagonist. A problem might arise in this experimentation, since theophylline, besides blocking purinergic receptors, at high concentrations also acts as a phosphodiesterase inhibitor (Butcher and Sutherland, 1962) and causes mobilization of intracellular calcium pools (Rall and West, 1963; McNeil et al., 1969). Therefore, the resolution of the question of the physiological significance of purinergic, presynaptic modulation probably must await the development of a more specific purinergic receptor antagonist.

STRUCTURE-ACTIVITY RELATIONSHIP STUDIES

Differences between presynaptic and postsynaptic alpha-adrenoceptors have been found in a number of tissues. Preferential stimulation of pre- or postsynaptic receptors may be of clinical importance (cf. Starke, 1977; Westfall, 1977). To date, one structure-activity relationship study of presynaptic purinergic receptors has been attempted.

Paton et al. (1978) have studied the structure-activity requirements for presynaptic purinergic activity in the rat vas deferens, in vitro. The presynaptic structural requirements included a primary or secondary amine function at C_6 of the purine ring and there was found to be little tolerance for major steric changes or substitutions on the sugar moiety (Paton et al., 1978). When these authors compared the presynaptic requirements with those of adenosine activity on coronary flow (Augers et al., 1971; Cobbin et al., 1974; Olsson and Patterson,

1976) and on relaxation of the isolated longitudinal muscle of the rabbit small intestine (McKenzie et al., 1977), they were found to be very similar. However, notable differences were seen with adenosine-N^1-oxide and 6-Δ^2-isopentenylaminopurine riboside which were inactive as coronary dilators but were active in the other systems (cf. Paton et al., 1978).

EFFECTS OF 2-CHLOROADENOSINE

Subsequent to the structural requirement study for presynaptic inhibition by adenosine (Paton et al., 1978), it was observed in this laboratory that 2-chloroadenosine was more potent and longer acting in inhibiting noradrenergic transmission than adenosine in the rat vas deferens. A high apparent potency and long duration of action of 2-chloroadenosine is found in many adenosine sensitive systems, for example: Depression of intestinal activity (Mihich et al., 1954; Satchell and Maguire, 1975); relaxation of blood vessels (Clarke et al., 1952; Gough et al., 1969; Angus et al., 1971) as well as other actions in various systems (Born, 1964; Constantine, 1965; Michal and Thorp, 1966; Gilman, 1974; Huang and Daly, 1974; Blume and Foster, 1975; Phillis and Kostopoulos, 1975; Huang and Drummond, 1976). It has been suggested that 2-chloroadenosine has a longer duration of action than adenosine because the former is resistant to enzymatic degradation by adenosine deaminase (Clarke et al., 1952); however, the high apparent potency of 2-chloroadenosine compared to adenosine has not been adequately explained. We have considered the possibility that uptake and metabolism of adenosine, which occurs in many or all cell types, may limit the potency of adenosine while not interfering with the action of

2-chloroadenosine.

UPTAKE OF ADENOSINE

Nucleoside transport mechanisms have generally been found to have the characteristics of facilitated diffusion, i.e., a carrier-mediated process with the following characteristics: Saturability, competition between related compounds and high temperature dependence. Furthermore, facilitated transport must be driven by the concentration gradient of the permeant and does not require metabolic energy. "Counter-flow" involves the uphill movement of a substrate for the same carrier, and this is the only time when movement of substrate occurs against a concentration gradient (cf. Stein, 1967).

The existence of a saturable uptake mechanism for adenosine in many cell types has been demonstrated by studying adenosine uptake kinetics as well as uptake inhibition by related permeants and specific inhibitors (Steck et al., 1969; Lieu et al., 1971; Oliver and Paterson, 1971; Plagemann, 1971; Mizel and Wilson, 1972; Olsson et al., 1972; Roos and Pfleger, 1972; Schrader et al., 1972; Taube and Berlin, 1972; Cass and Paterson, 1973; Olsson and Paterson, 1976). In some cells the uptake carrier has been shown to be specific for adenosine (Plagemann, 1971; Plagemann and Richey, 1974; Cass and Paterson, 1977; Paterson et al., 1977a; Paterson et al., 1977b), while in other cells the carrier is less selective and accepts a variety of purines and pyrimidines (Oliver and Paterson, 1971; Taube and Berlin, 1972; Cass and Paterson, 1973). At high concentrations, adenosine may also enter cells by passive diffusion (Roos and Pfleger, 1972; Schrader et al., 1972; Paterson et al., 1977a).

INHIBITORS OF ADENOSINE UPTAKE

Many adenosine analogs inhibit adenosine transport into cells in a reversible, competitive manner. Long acting, competitive antagonists of adenosine transport have also been found in the form of thioethers of purine or of 2-aminopurine ribosides (Paterson and Simpson, 1965, 1966, 1967). Paterson and Oliver (1971) showed that p-nitrobenzyl-thioguanosine (NBTG) caused a competitive, long lasting inhibition of carrier-mediated uptake and efflux of uridine in red cells, where uridine and adenosine utilize the same transport carrier (Oliver and Paterson, 1971; Cass and Paterson, 1973). HNBTG also has been shown to inhibit adenosine uptake in the heart (Olsson et al., 1972).

Dipyridamole is well known as an inhibitor of adenosine uptake. Nucleoside transport has been shown to be inhibited by this agent in various cells (Bunag et al., 1964; Scholtissek, 1968; Plagemann, 1971; Roos and Pfeger, 1972; Schrader et al., 1972) including the heart (Kolassa et al., 1970; Olsson et al., 1972; Hopkins, 1973) and the guinea-pig taenia coli (Satchell et al., 1972). The action of adenosine has been shown to be potentiated by dipyridamole in a number of preparations (Stafford, 1966; Satchell et al., 1972; Bell, 1974; Satchell and Burnstock, 1975; Kalsner, 1975; Colman, 1976).

Low concentrations of dipyridamole were claimed to potentiate the responses of the guinea-pig taenia coli to both purinergic nerve stimulation and ATP while not altering the inhibitory responses to sympathetic nerve stimulation and noradrenaline (Satchell et al., 1972). In this study it was presumed that dipyridamole acted by inhibition of adenosine uptake, thus effectively increasing its extracellular concentration and perhaps reduce the breakdown of ATP, the presumed transmitter,

by a feedback mechanism. This has been taken as evidence supporting the purinergic nerve hypothesis. Jager (1976) studied the effects of dipyridamole on the guinea-pig taenia coli using single and double sucrose gap methods to measure membrane potential and membrane resistance and concluded that dipyridamole does not potentiate nonadrenergic relaxations by interfering with the neuromuscular transmission, which has been assumed to be purinergic. Furthermore, Baer et al. (1977) have re-investigated the above claims by Satchell et al. (1972) quantitatively and found that dipyridamole did not affect the frequency-response curve in the guinea-pig taenia coli, concluding therefore that neither dipyridamole nor HNBTG have any use in the study of non-adrenergic inhibitory nerves in smooth muscle.

Problems arise when using a drug which has other non-specific actions. It has been suggested that dipyridamole may inhibit adenosine deaminase (Bunag et al., 1964) and phosphodiesterase (Sneft, 1968). At the concentrations used by others (Hopkins and Goldie, 1971; Olsson et al., 1972; Schrader et al., 1972), dipyridamole did not alter either adenosine kinase or adenosine deaminase activity. Dipyridamole has also been shown to inhibit uptake of phosphates, certain sugars and choline in various cells (Plagemann and Roth, 1969; Renner et al., 1972). Recently, it has been suggested that dipyridamole releases noradrenaline from noradrenergic nerves by a mechanism which differs from stimulation of nerves or from the action of tyramine (Himari and Taira, 1976).

At present, no non-specific effects of HNBTG have been reported, and this class of compound may prove to be a more useful tool and preferable to dipyridamole in functions involving adenosine uptake.

FATE OF ADENOSINE FOLLOWING UPTAKE

Generally, metabolism of adenosine subsequent to uptake results in either phosphorylated or deaminated products. Adenosine kinases phosphorylate adenosine to AMP which may then be further phosphorylated to ATP. For example, in the heart (Jacob and Berne, 1960; Liu and Feinberg, 1971; Olsson et al., 1972) and in the guinea-pig vas deferens (Rowe et al., 1975) incubation of tissues with radioactive adenosine mainly results in labelling of the adenine nucleotide pools. Alternatively, adenosine can be deaminated to inosine by adenosine deaminase. Both inosine and hypoxanthine, which is formed from inosine by nucleoside phosphorylase, are generally pharmacologically inactive. The Km for adenosine kinase has been found to be less than the Km for adenosine deaminase; thus, Schrader et al. (1972) have suggested that phosphorylation of adenosine normally occurs at lower substrate concentrations than deamination. Adenosine, as such, normally does not appear in cells (cf. Schrader et al., 1972), indicating that metabolism is complete.

It has been suggested that adenosine entry and deamination are separate events (Taube and Berlin, 1972) on the basis of the following. The Km for adenosine uptake (0.01 mM) and deamination (0.073 mM) were different. Also, the specificities of the transport system and of adenosine deaminase were not the same (i.e., pyrimidine nucleosides were poor substrates for adenosine deaminase but good ones for the transport carrier).

INHIBITORS OF ADENOSINE DEAMINASE

Recently, very effective inhibitors of mammalian adenosine deaminase

have become available. Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) is a reversible inhibitor with a K_i of about 10^{-8}M (Schaeffer and Schwender, 1974). An even more potent, tight binding inhibitor of mammalian adenosine deaminase is 2'-deoxycoformycin with a K_i of less than 10^{-10}M (Woo et al., 1974; Cha et al., 1975). Both, EHNA (Cohen and Plunkett, 1975; Plunkett and Cohen, 1975) and 2'-deoxycoformycin (Cass and AuYeung, 1976; Johns and Adamson, 1976; LePage et al., 1976) have been shown to potentiate the cytotoxic effects and antitumor activity of several adenosine analogs which are metabolized by adenosine deaminase.

Whether the actions of adenosine on an extracellular adenosine receptor are potentiated by adenosine deaminase inhibitors has not been examined. Such a potentiation would provide evidence supporting the hypothesis that the action of adenosine is masked by metabolism (Satchell and Burnstock, 1975).

RATIONALE

From the introduction it is evident that adenosine inhibits noradrenergic transmission and that this inhibition is mediated specifically via adenosine-receptors. These receptors appear to be located on the extracellular surface of the noradrenergic nerve terminal.

Subsequent to a study by Paton et al. (1978) of the structure-activity requirements for presynaptic inhibition by adenosine, it was observed in this laboratory that 2-chloroadenosine was more potent and longer acting than adenosine in inhibiting nerve-mediated responses of rat vas deferens, in vitro. In addition, a number of other 2-substituted adenosine derivatives have been shown to possess these properties,

in other adenosine sensitive systems. There was no explanation for the high apparent potencies of these compounds at the onset of this study. We have compared the effects of various 2-substituted adenosine derivatives to those of adenosine in rat vas deferens.

The following questions were asked:

(1) Do 2-substituted adenosine derivatives inhibit noradrenergic transmission presynaptically?

(2) If so, do these compounds act via specific adenosine-receptors?

(3) What is the role of uptake and deamination in determining apparent potencies of adenosine and 2-chloroadenosine?

METHODS

ANIMALS

Male Wistar rats weighing 150 to 250 g were employed in all experiments. No more than 6 rats were housed in one cage. Two rats were chosen at random on each experimental day. Environmental conditions were kept constant. The temperature was maintained at 21°C and the lighting cycle was 12 h light and 12 h darkness. Commercial rat food and water were supplied ad libitum.

MEASUREMENT OF ISOMETRIC CONTRACTIONS

The rats were killed by cervical dislocation and exsanguination. The abdomens were opened and the vasa deferentia were excised and placed in Krebs solution. Vasa deferentia were then carefully dissected free from adhering tissue. The middle portion of the rat vas deferens was used, consisting of equal lengths of urethral (think) and testicular (thin) portions. The urethral end of the tissue was attached to a platinum hook electrode at the bottom of the tissue bath. The testicular end was attached to a transducer. The tissues were mounted in a 5 ml tissue bath containing Krebs solution (pH 7.4) which was aerated with 95% O_2/5% CO_2 and maintained at 37°C (Fig. 1). The rat vas deferens was maintained under 0.5 g resting tension throughout all experiments. There was a stabilization period of at least 30 minutes before the start of each procedure.

Contraction of the muscle in response to electric field stimulation or to drugs were measured as changes in isometric tension with Grass (FT03C) force displacement transducers and displayed on a Grass (Model 5D) Polygraph or a Beckman (Type RB) Dynograph. Electrical field stimulation of the preparation was elicited with a Grass (Model

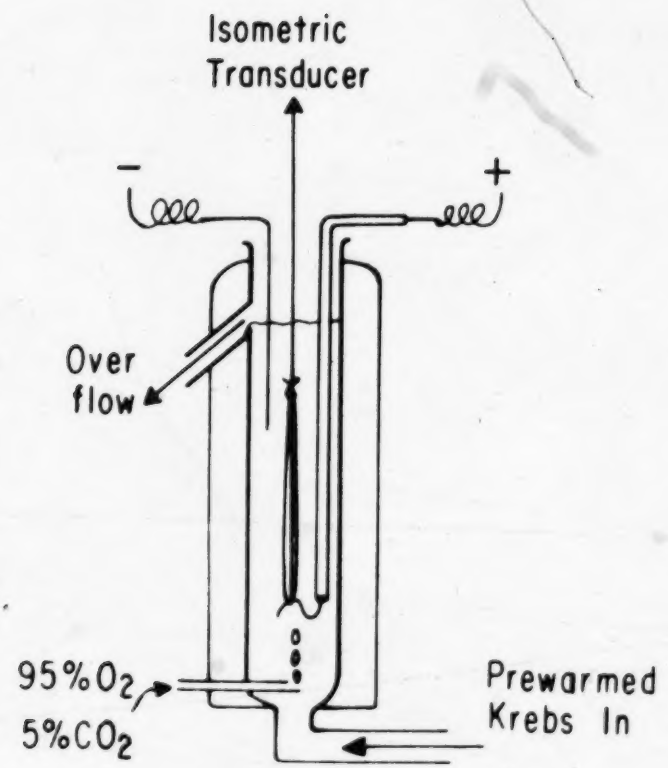

FIG. 1 Schematic illustration of contractility apparatus.

SM6C) stimulator by passing biphasic pulses of 1 ms duration and a supramaximal voltage (100 volts) between platinum electrodes located at the top and bottom of the tissue bath.

SOLUTIONS AND DRUGS

The Krebs solution consisted of analytical grade chemicals obtained from Fisher Scientific Company. It had the following composition: NaCl, 116 mM; KCl, 5.4 mM; $CaCl_2$. 2.5 mM; $MgCl_2$, 1.2 mM; NaH_2PO_4, 1.2 mM; $NaHCO_3$, 22.0 mM; D-glucose, 11.2 mM. The solution was made in distilled deionized water, equilibrated with 95% O_2/5% CO_2 and maintained at 37°C .

Drugs and their sources were: Adenosine, atropine sulphate, 2-chloroadenosine, indomethacin, (-)-noradrenaline bitartarate and theophylline (Sigma Chemical Co.); 2-bromoadenosine*, 2-fluoroadenosine* (Southern Research Institute); 2-amino -6-[(2-hydroxy-5-nitro)benzylthio]-9-β-D-ribofuranosylpurine* (HNBTG, Dr. A.R.P. Paterson, University of Alberta Cancer Research Unit); 2'-deoxycoformycin* (Dr. G.A. LePage, University of Alberta Cancer Research Unit); dipyridamole* (Boehringer-Ingelheim); 2-hydroxyadenosine* (Dr. J.F. Henderson, University of Alberta Cancer Research Unit); phenoxybenzamine HCl* (Smith, Kline and French) and dimethylsulfoxide (DMSO, Fisher Scientific Co.). Compounds indicated by an asterisk were kindly donated by the individuals or firms shown.

Stock solutions (10^{-2}M) of 2-bromoadenosine, 2-chloroadenosine, 2-fluoroadenosine and 2-hydroxyadenosine and (10^{-1}M) adenosine and 2-aminoadenosine, were made in dimethylsulfoxide (DMSO) and stored frozen. DMSO was used because of the low solubility of the 2-substituted adenosine derivatives in water. DMSO (1% v/v) alone did not alter

significantly the twitch or sustained responses of rat vas deferens to field stimulation. Furthermore, inhibition of tissue responses to field stimulation by adenosine (10^{-4}M) were the same when adenosine was dissolved in DMSO. 2'-Deoxycoformycin (60 ug/ml) stock solution was obtained in saline solution from Dr. G.A. LePage. Aliquots of 0.2 ml of these drugs were stored frozen.

A stock solution of noradrenaline (10^{-2}M) in 0.1 mM HCl was stored frozen. Dipyridamole was dissolved in the minimum volume of 1 M HCl. Indomethacin was prepared in equimolar sodium carbonate solution and was made up fresh every 3 h.

FREQUENCY-RESPONSE DETERMINATIONS

Frequency-response curves were determined from the urethral portion, testicular portion and middle portion (consisting of equal lengths of urethral and testicular portions) of rat vas deferens in the absence and in the presence of adenosine. These experiments indicated which portion(s) of the tissue and what frequency of stimulation were to be utilized in the present study.

INHIBITION BY ADENOSINE AND 2-SUBSTITUTED ADENOSINE DERIVATIVES

The effects of increasing concentrations of 2-chloroadenosine, 2-hydroxyadenosine, 2-bromoadenosine, 2-fluoroadenosine, 2-aminoadenosine and adenosine on twitch and sustained responses of rat vas deferens to electrical field stimulation were compared to the effects of these compounds on submaximal responses to exogenous noradrenaline. This indicated whether these drugs were acting pre- and/or postsynaptically.

EVALUATION OF DRUG EFFECTS

Dose-response curves of inhibition by the 2-substituted adenosine derivatives of responses of rat vas deferens to nerve stimulation were compared on a paired basis with the inhibition caused by adenosine. Thus, relative potencies and durations of action of the drugs were determined.

Dose-response curves of the inhibition by adenosine and various 2-substituted adenosine derivatives of nerve-mediated responses of rat vas deferens were done on a paired basis in the absence and in the presence of theophylline, phenoxybenzamine, atropine, indomethacin or dipyridamole. In other experiments, the effects of adenosine and 2-substituted adenosine derivatives were compared on a paired basis in normal tissues and tissues pretreated with HNBTG and/or 2'-deoxycoformycin or with both of these drugs and theophylline.

On a paired basis with normal tissues, the effects of various drugs (HNBTG, dipyridamole, theophylline and 2'-deoxycoformycin) on frequency-response curves of rat vas deferens were determined to check for non-specific effects of these agents. Further, frequency-response curves from normal rat vas deferens were compared on a paired basis with tissues pretreated with both HNBTG and 2'-deoxycoformycin to determine whether adenosine has a physiological modulatory role on noradrenergic transmission in this tissue.

EVALUATION OF RESULTS

(a) Frequency-response determinations

Frequency-response curves were constructed by stimulating the rat vas deferens with biphasic pulses of constant duration and voltage

(1 ms duration and 100 volts) while increasing the frequency of stimulation until attainment of maximum responses for both twitch and sustained responses. A 5 minute time cycle was followed, stimulating the tissue until achievement of the full response (which took about 30 s). Results were expressed as a percentage of the maximum for both the twitch and sustained responses of rat vas deferens to field stimulation. Also, control maximum responses were compared to experimental maximum responses.

(b) Electrical field stimulation

Reproducible, submaximal contractions of rat vas deferens were elicited every 5 minutes for 30 seconds to electrical field stimulation (biphasic pulses of 1 ms duration at supramaximal voltage and 5 Hz). The response obtained 60 seconds following the addition of adenosine or the 2-substituted adenosine derivatives was expressed as a percentage of the preceding control response (Fig. 2). The percentage inhibition was calculated for both the twitch and sustained responses and plotted against increasing concentrations of drug. Following washing the tissue, the stimulation was repeated, until reproducible responses similar to the initial controls were obtained.

(c) Exogenous noradrenaline

Reproducible contractions of rat vas deferens were elicited every 5 minutes for 30 seconds to exogenous noradrenaline (5×10^{-6}M) which gave responses of about 50% of the maximum. Only sustained contractions resulted from noradrenaline application at this concentration. Experiments were performed and evaluated in the same manner as described for electrical field stimulation.

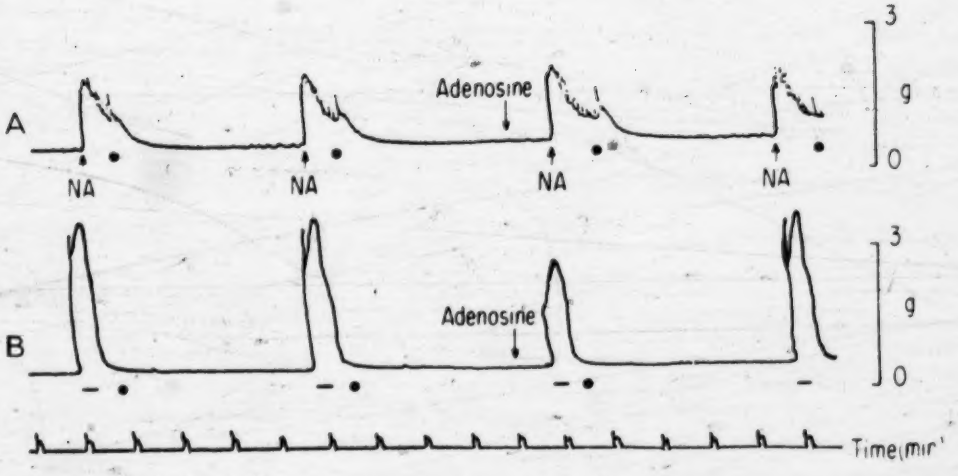

FIG. 2 Effect of adenosine (10^{-4}M) on responses of rat vas deferens to exogenous noradrenaline (5×10^{-6}M) (A), and on responses to electrical field stimulation (5 Hz, 30 s) (B).

STATISTICAL ANALYSIS OF DATA

Results are expressed as mean ± standard error of the mean (S.E.M.). Significance levels for the difference between groups or slopes were estimated using Student's paired 't' test and the difference was judged to be significant when $P < 0.05$. ID_{50} values and slopes were extrapolated by computer regression analysis of responses obtained between 20-80% inhibition on the log concentration vs response curves.

RESULTS

RESPONSES OF RAT VAS DEFERENS TO ELECTRICAL FIELD STIMULATION

Electrical field stimulation of the urethral portion of rat vas deferens mainly resulted in twitch responses at all frequencies of stimulation (Fig. 3a); therefore, twitch responses appear to originate from this portion of the tissue. On the other hand, electrical field stimulation of the testicular portion of the rat vas deferens mainly resulted in sustained responses at all frequencies of stimulation (Fig. 3b); therefore, sustained responses appear to originate from this portion of the tissue. As expected, electrical field stimulation of the middle portion of the vas deferens which consisted of equal lengths of urethral and testicular portions of the tissue resulted in both twitch and sustained responses at higher frequencies of stimulation. However, at low frequencies of stimulation, only twitch responses were elicited from the middle portion of the vas deferens (Fig. 3c). This may be a result of the morphology of innervation of the tissue, sustained responses being elicited by stimulation of extrajunctional alpha-adrenoceptors.

FREQUENCY-RESPONSE DETERMINATIONS

(A) Middle portion of rat vas deferens

The log frequency-response curves for both the twitch and sustained responses of the middle portion of the rat vas deferens to electrical field stimulation are shown in Figure 4. The slope of the twitch frequency-response curve decreased from 5 to 20 Hz but increased again from 20 to 100 Hz. Thus, the frequency-response curve appears biphasic in shape. The shape of the frequency-response curve for the sustained contraction was also biphasic in nature; however, the maximum occurred at 50 Hz.

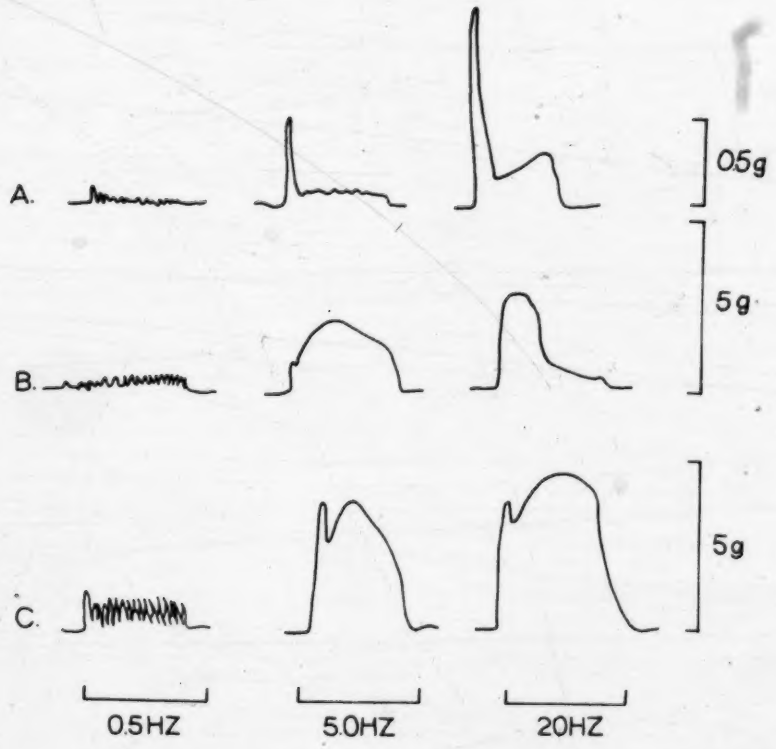

FIG. 3 Twitch and sustained responses to field stimulation (0.5, 5.0 and 20.0 Hz, 30 s) from urethral (A), testicular (B), and middle (C) portions of rat vas deferens.

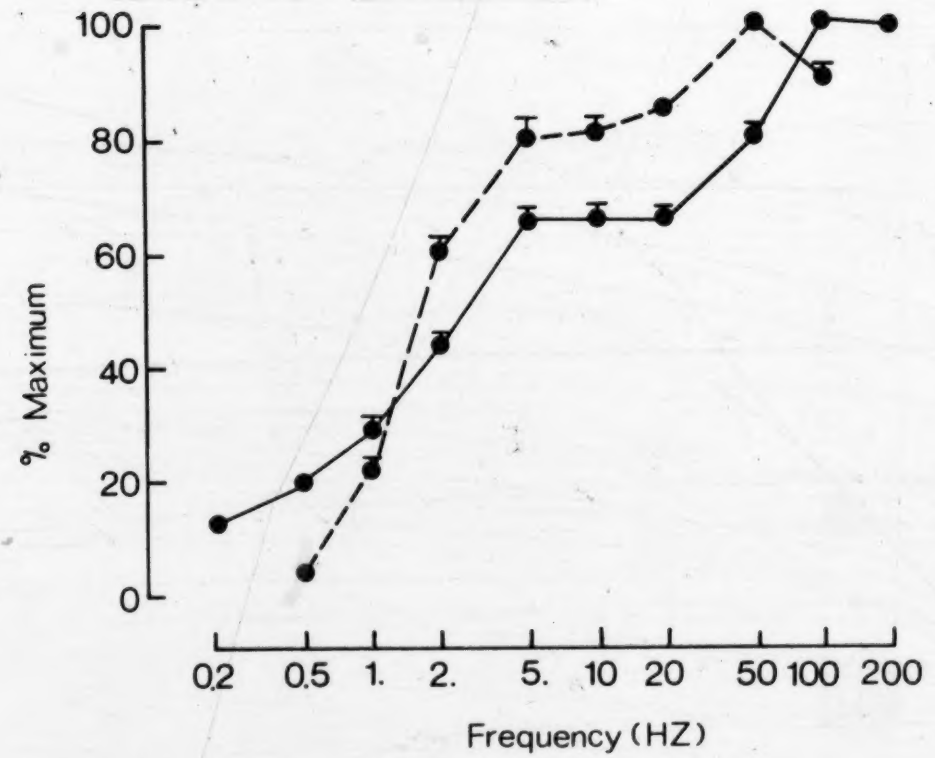

FIG. 4 Plot of frequency-response curves of twitch (——) and sustained (- - - -) responses of the middle portion of the rat vas deferens. Results are expressed as mean % maximum ± S.E.M., n = 6.

The secondary increase in the frequency-response curves may have been due to direct muscle stimulation, therefore, the effect of TTX, a blocker of action potentials in neurons, was determined on responses of the rat vas deferens to electrical field stimulation. TTX abolished all responses of the tissue to electrical field stimulation up to 100 Hz, however, at 200 Hz sustained responses were elicited which were probably due to direct muscle stimulation. These results indicate that the secondary increase in the slope of the frequency-response curves was not due to direct muscle stimulation.

(B) Separate portions of the rat vas deferens

The frequency-response determinations from the urethral portion of the rat vas deferens is shown in Figure 5, and the frequency-response determination from the testicular portion of this tissue in Figure 5. A linear relationship resulted from plotting the frequency vs % maximum twitch response of the urethral portion of the vas deferens with a maximum occurring at 100 Hz. On the other hand, an S-shaped curve of frequency vs % maximum sustained responses with a maximum occurring at 20 Hz was elicited from the testicular portion. These results suggest that the biphasic shape of the frequency-response curves from the middle portion of the vas deferens may be due to an interaction between the two portions of this tissue.

FREQUENCY-DEPENDENCE OF INHIBITION OF ADENOSINE

The effect of adenosine (10^{-4}M) on the frequency-response curves of both the twitch and sustained responses of the middle portion of the rat vas deferens is shown in Figure 6 a and b. Three observations can be

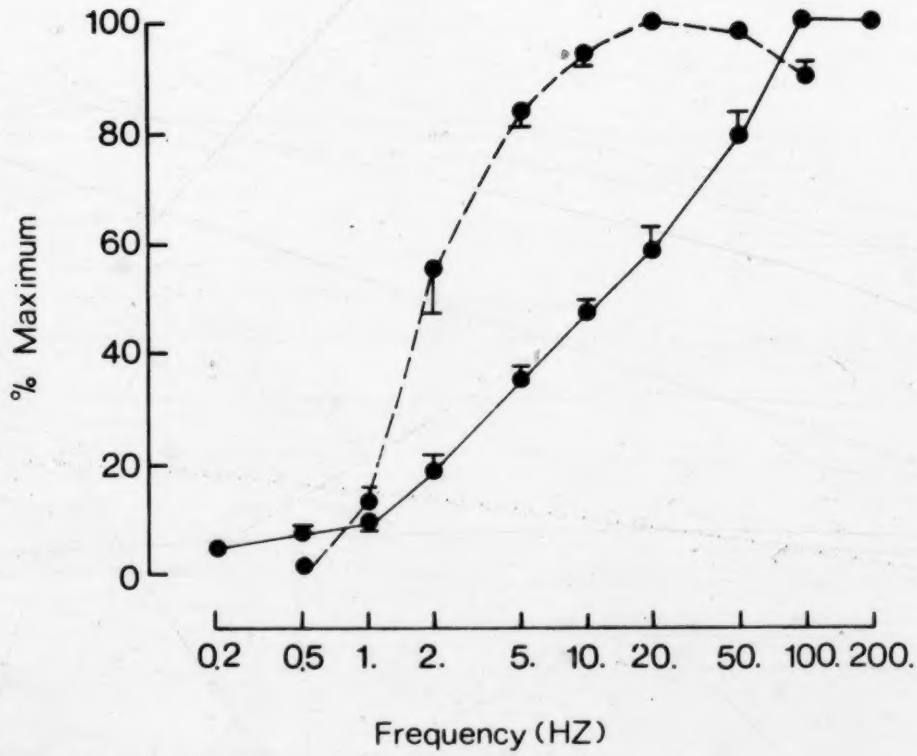

FIG. 5 Plot of frequency-response curves of twitch (——) responses from the urethral portion and sustained (- - -) responses from the testicular portion of the rat vas deferens. Results are expressed as mean % maximum ± S.E.M., n = 6.

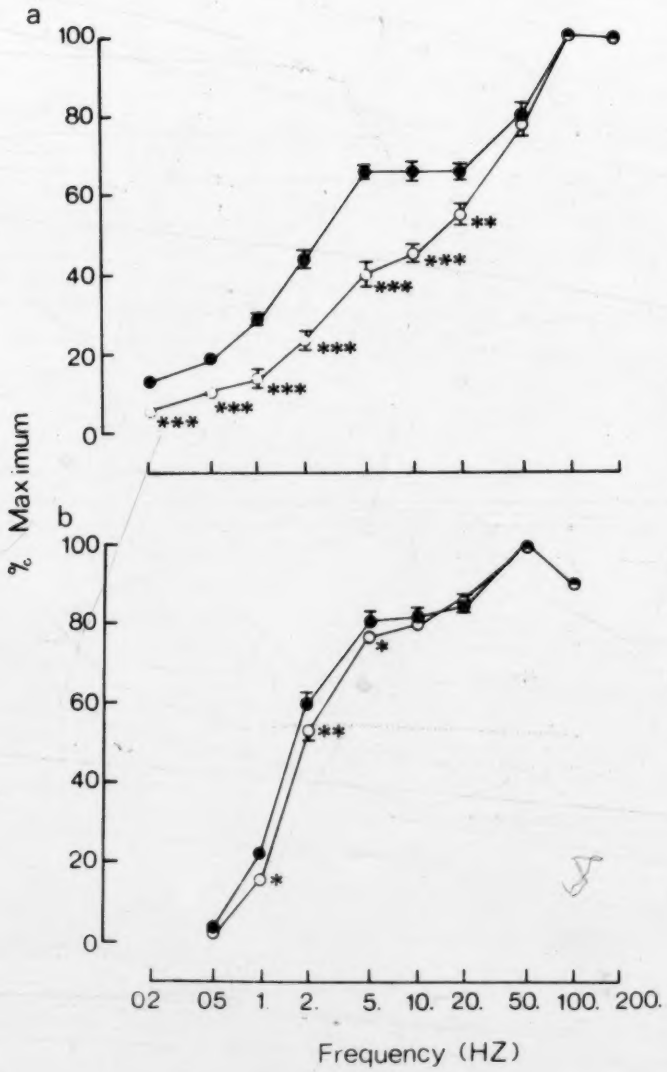

FIG. 6 Effect of adenosine (0; 10^{-4}M) on frequency-response curves of twitch (a) and sustained (b) responses of the middle portion of the rat vas deferens. Results are expressed as mean % maximum ± S.E.M.; n = 6. Asterisks represent the significance of the difference from normal tissues; * $0.05 > P > 0.01$; ** $0.01 > P > 0.001$; *** $P < 0.001$.

made from this experiment. Adenosine inhibited both twitch and sustained responses of the tissue to electrical field stimulation with the former being more sensitive to the inhibition by adenosine than the latter. Also, both in the case of the twitch responses and of the sustained responses, adenosine inhibited these to a greater extent at lower frequencies than at higher frequencies of stimulation. In this respect, adenosine had no inhibitory activity at frequencies above 20 Hz for the twitch or above 5 Hz for the sustained responses.

Twitch responses from the urethral portion and sustained responses from the testicular portion of rat vas deferens were also inhibited by adenosine (10^{-4}M) in a similar manner (Fig. 7 a and b). Once again, both of these responses were inhibited to a greater extent at lower than at higher frequencies of stimulation and twitch responses were more sensitive than sustained responses to the inhibition by adenosine. Twitch responses from the urethral portion were inhibited at all frequencies of stimulation; however, sustained responses from the testicular portion were not inhibited at frequencies above 10 Hz. Therefore, the inhibitory activity of adenosine was not obscured by utilizing the middle portion of the rat vas deferens.

The observation that adenosine inhibited low frequency stimulation to a greater extent than high frequency stimulation was examined further by studying the inhibitory effects of increasing concentrations of adenosine on twitch responses of the rat vas deferens due to electrical field stimulation at 5 Hz (30 s stimulation every 5 min) or at 0.03 Hz (constant stimulation) (Fig. 8). It can be seen that the inhibition of twitch responses by adenosine was frequency-dependent, with low frequency twitch responses being more sensitive to the inhibitory action of adenosine than higher frequency twitch responses.

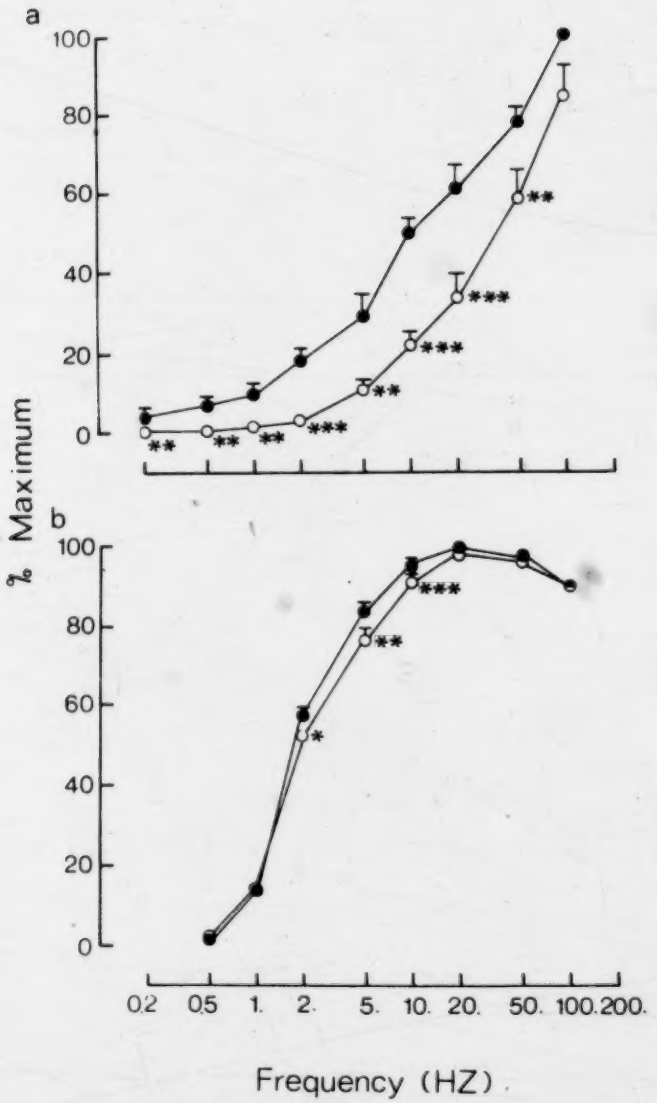

FIG. 7 Effect of adenosine (0; 10^{-4}M) on frequency-response curves of twitch (a) responses from the urethral portion and sustained (b) responses from the testicular portion of the rat vas deferens. Results are expressed as mean % maximum ± S.E.M., n = 6. Asterisks represent the significance of the difference from normal tissues; * $0.05 > P > 0.01$; ** $0.01 > P > 0.001$; *** $P < 0.001$.

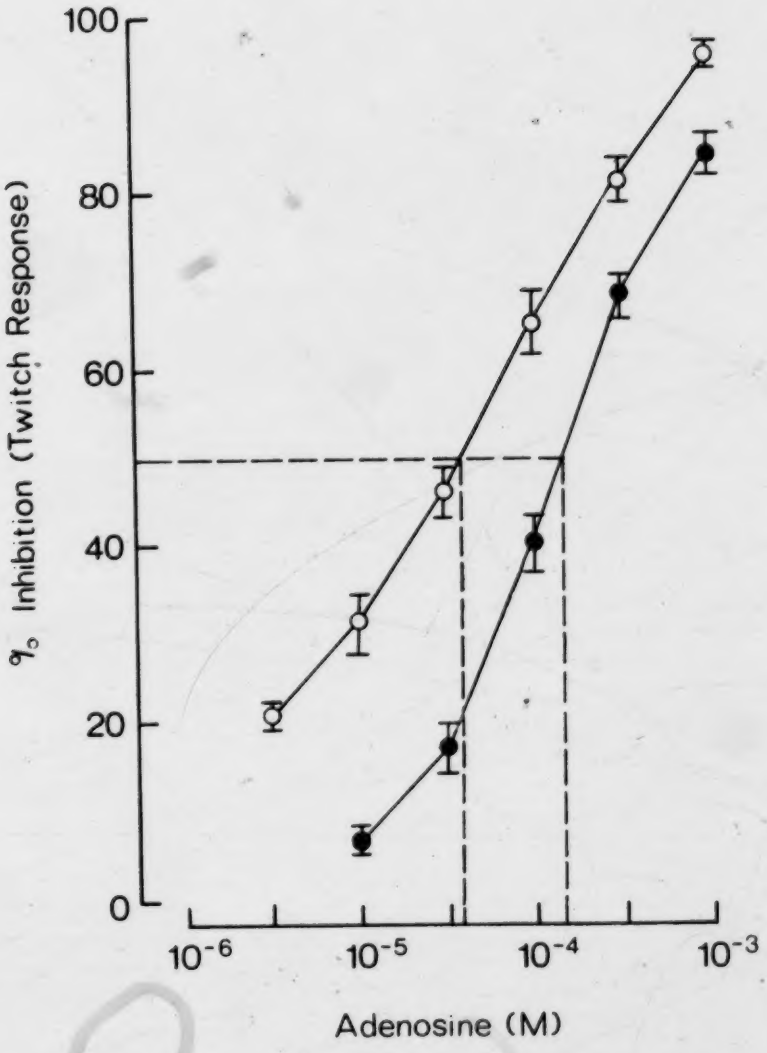

FIG. 8 Inhibition by adenosine of twitch responses of rat vas deferens to field stimulation at low frequency (0.03 Hz, 30 s) (O) or at higher frequency (5 Hz, 30 s) (●) of stimulation Results are expressed as mean inhibition ± S.E.M., n = 6.

EFFECTS OF ADENOSINE AND 2-SUBSTITUTED ADENOSINE DERIVATIVES ON CONTRACTILITY

(A) Effects on muscle tension

The rat vas deferens *in vitro* is a quiescent muscle exhibiting no spontaneous activity. Adenosine and the 2-substituted adenosine derivatives had no effect on the resting tension of the tissue *per se* in any of the concentrations tested.

(B) Effects on responses to electrical field stimulation

Adenosine (3-1000 uM) and the 2-substituted adenosine derivatives (1-100 uM) inhibited both twitch and sustained responses of the rat vas deferens to electrical field stimulation (5 Hz, 30 s every 5 min) in a concentration dependent manner (Fig. 9 a and b). The order of potency of these compounds in inhibiting responses to field stimulation was as follows: 2-Chloroadenosine $\geqslant$ 2-hydroxyadenosine $\geqslant$ 2-bromoadenosine $\geqslant$ 2-fluoroadenosine > 2-aminoadenosine = adenosine. All of the 2-substituted adenosine derivatives tested except 2-aminoadenosine were more potent than adenosine. The twitch response exhibited greater sensitivity to the inhibitory action of these compounds than did the secondary sustained response.

(C) Effects on responses to exogenous noradrenaline

Motor responses of the rat vas deferens elicited by exogenous noradrenaline (5×10^{-6}M for 30 s every 5 min) were only slightly inhibited by adenosine and the 2-substituted adenosine derivatives (Fig. 9c). Further, the effects of adenosine and the 2-substituted adenosine

FIG. 9 Effect on twitch (a) and sustained (b) responses of rat vas deferens to field stimulation (5 Hz, 30 s) and to exogenous noradrenaline (5×10^{-6}M) (c) of 2-chloroadenosine (■), 2-hydroxyadenosine (♦), 2-bromoadenosine (▼), 2-fluoroadenosine (▲), 2-aminoadenosine (⬢) and adenosine (●). Results are expressed as mean inhibition ± S.E.M., n=6, except adenosine, n=30. Asterisks represent the significance of the difference of effects on the sustained responses and on responses to exogenous noradrenaline; * $0.05 > P > 0.01$; ** $0.01 > P > 0.001$; *** $P < 0.001$.

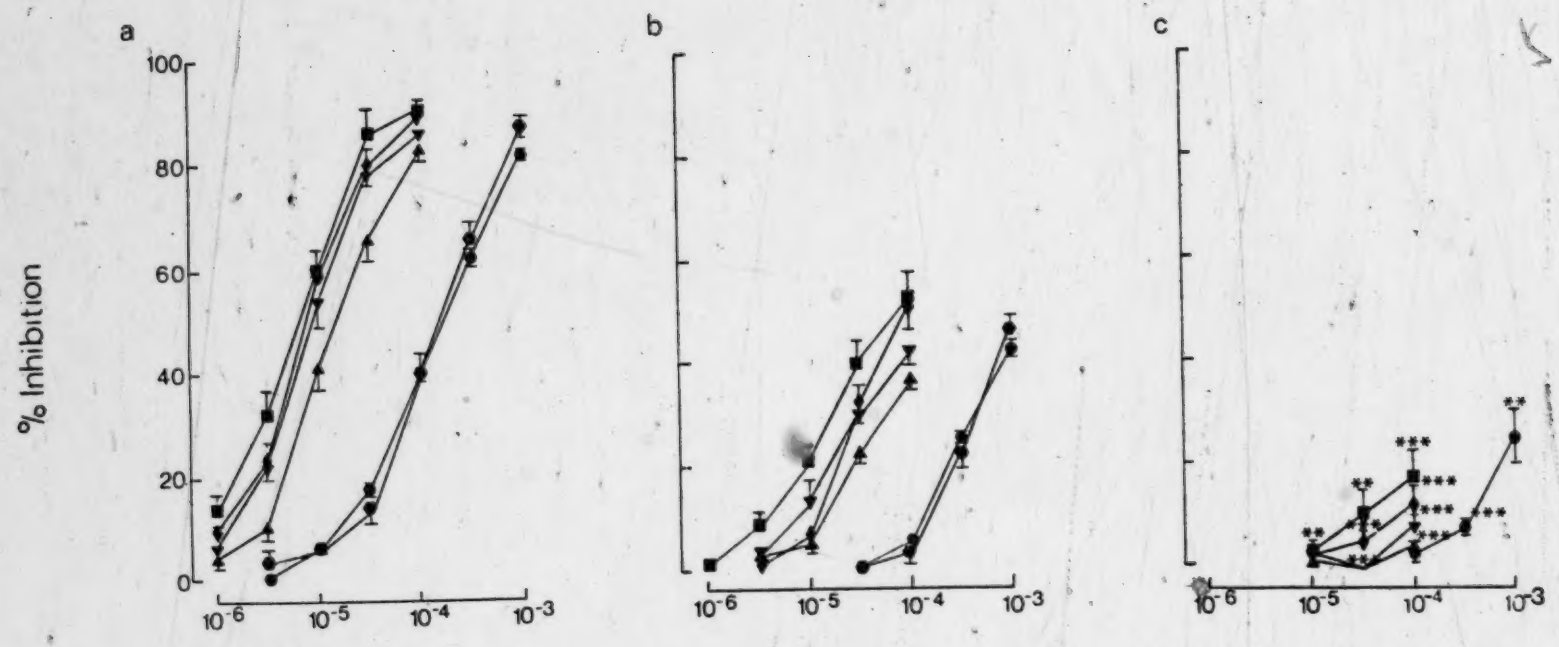
a
b
c
% Inhibition
100
80
60
40
20
0
10^{-6}
10^{-5}
10^{-4}
10^{-3}
Log Concentration (M)

FIG. 9

TABLE I Relative potencies of adenosine and 2-substituted adenosine derivatives. Experiments done on a paired basis within tissues with adenosine dose-response curve done before that of the derivative; n=6, except adenosine n=30.

DRUG	TWITCH IC_{50} (M)	±	(S.E.M.)
Adenosine	1.7×10^{-4}		(0.1)
2-Aminoadenosine	1.6×10^{-4}		(0.2)
2-Fluoroadenosine	1.6×10^{-5}		(0.5)
2-Bromoadenosine	9.2×10^{-6}		(1.7)
2-Hydroxyadenosine	7.9×10^{-6}		(1.8)
2-Chloroadenosine	7.4×10^{-6}		(1.3)

derivatives on responses of the rat vas deferens to exogenously applied noradrenaline were significantly different from the inhibitory effects of these compounds on either twitch or sustained responses of tissues to electrical field stimulation. Therefore, if noradrenaline is the functionally important neurotransmitter in the rat vas deferens then adenosine and its derivatives appear to have a predominately presynaptic site of action, although these compounds also act postsynaptically.

EFFECTS OF THEOPHYLLINE ON CONTRACTILITY OF RAT VAS DEFERENS

Theophylline is a commonly used antagonist of purinergic receptors, inspite of its nonspecific actions at high concentrations. In order to find an effective dose of theophylline with minimal direct effects the inhibition by increasing concentrations of theophylline applied 60 seconds prior to electrical field stimulation (5 Hz for 30 s every 5 min) of rat vas deferens was studied (Fig. 10). The threshold concentration of inhibitory activity of theophylline was found to be about 10^{-4}M. At higher concentrations, theophylline inhibited both twitch and sustained responses in a concentration dependent manner, as did adenosine and the 2-substituted adenosine derivatives with the twitch responses being more sensitive than the secondary sustained responses. There was no difference in inhibitory activity of theophylline (10^{-3}M) on responses of tissues to electrical field stimulation and on responses of tissues to exogenously applied noradrenaline (5×10^{-6}M for 30 s every 5 min), therefore, unlike the inhibition by adenosine and derivatives, this effect of theophylline appeared to be mediated postsynaptically.

The ID_{50} for adenosine inhibitory activity in the rat vas deferens

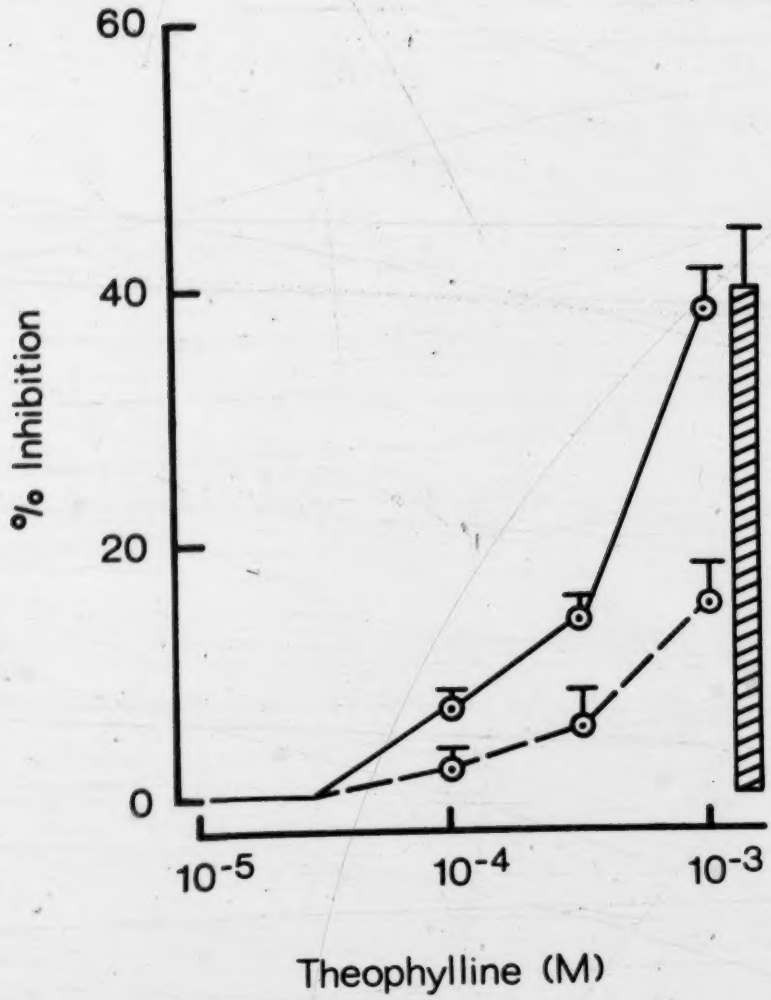

FIG. 10 Inhibition by theophylline of twitch (——) and sustained (---) responses of rat vas deferens to field stimulation (5 Hz, 30 s) and to exogenous noradrenaline (5×10^{-6}M) (▨). Results are expressed as mean inhibition ± S.E.M., n=6.

was found to be about 3×10^{-4}M. Therefore, a high concentration of theophylline was required for competition with adenosine. Although 10^{-4}M theophylline was the threshold concentration for inhibitory activity, this concentration did not significantly alter frequency-response determinations of twitch or sustained responses. Therefore, this concentration of theophylline was utilized in subsequent studies with this purinergic receptor antagonist.

ANTAGONISM BY THEOPHYLLINE OF EFFECTS OF 2-SUBSTITUTED ADENOSINE DERIVATIVES

The inhibition by 2-chloroadenosine, 2-hydroxyadenosine, 2-bromo-adenosine and 2-fluoroadenosine of twitch and sustained responses of rat vas deferens to electrical field stimulation were significantly antagonized by theophylline (10^{-4}M) (Fig. 11 a, b, c and d). Furthermore, theophylline caused a parallel shift to the right of the dose-response curves of these compounds. Since maximum inhibition was not achieved, because of solubility characteristics of the 2-substituted adenosine derivatives, it was not possible to determine whether theophylline also depressed the maximum inhibition by these compounds. These results suggest that the 2-substituted adenosine derivatives act via a purinergic receptor.

The effect of theophylline on the inhibitory activity of adenosine in the rat vas deferens is discussed later.

EFFECTS OF ALPHA-ADRENOCEPTOR OR MUSCARINIC RECEPTOR BLOCKADE ON INHIBITION BY 2-CHLOROADENOSINE

Presynaptic alpha-adrenoceptors and muscarinic receptors are

FIG. 11 Inhibition by 2-chloroadenosine (a), 2-hydroxyadenosine (b), 2-bromoadenosine (c) and 2-fluoroadenosine (d) of twitch (——) and sustained (---) responses of rat vas deferens to field stimulation (5 Hz, 30 s) in normal Krebs (■ , ♦ , ▼ , ▲) and in the presence of theophylline (100 μM; □ , ◊ , ▽ , △). Results are expressed as mean inhibition ± S.E.M., n=6. Asterisks represent the significance of the difference of inhibition from normal Krebs; * $0.05 > P > 0.01$; ** $0.01 > P > 0.001$; *** $P < 0.001$.

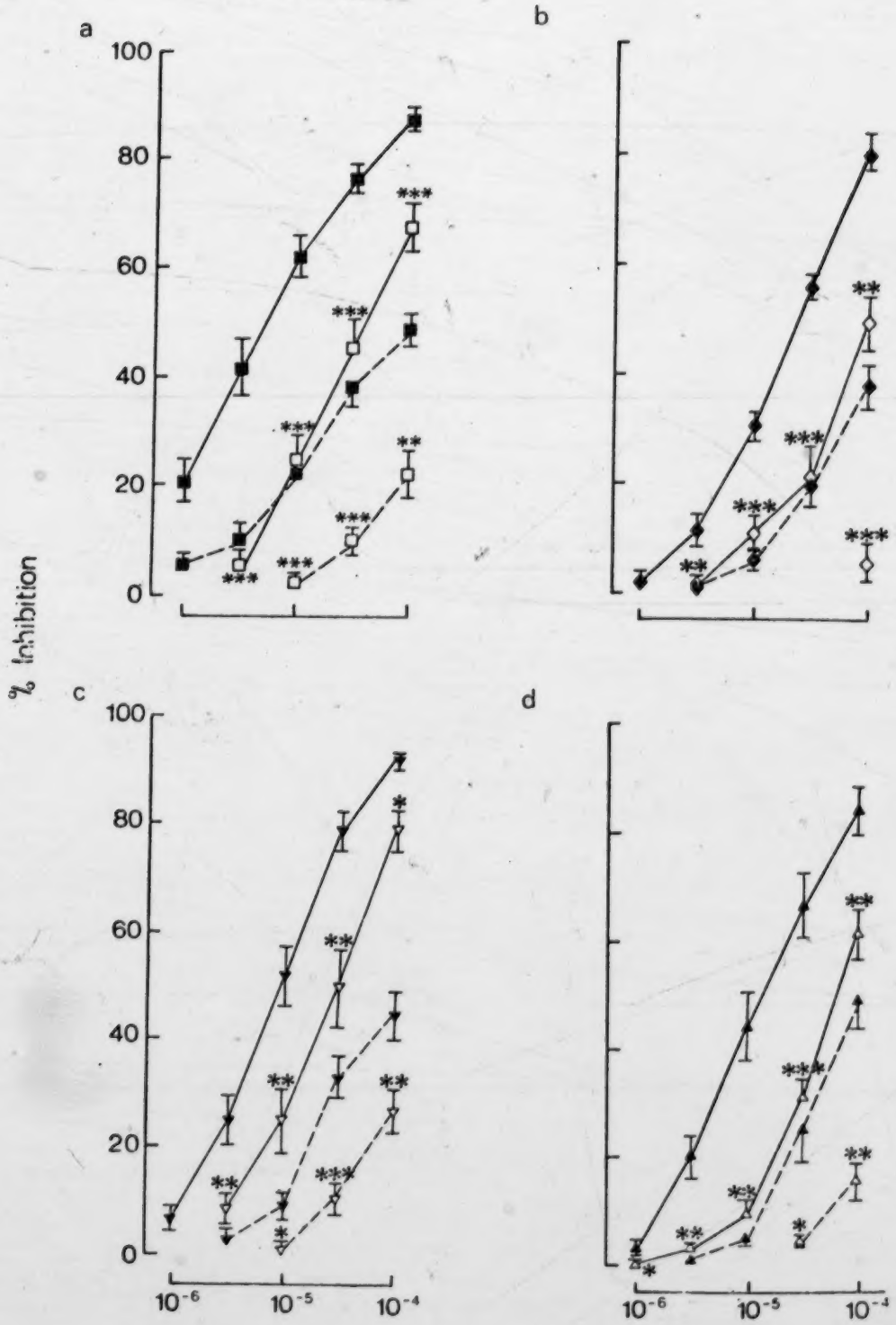
a
b
c
d
100
80
60
40
20
0
% Inhibition
10^{-6}
10^{-5}
10^{-4}
Log Concentration (M)

FIG. 11

present and may be of functional significance in the vas deferens. Since neither phenoxybenzamine (5×10^{-6}M) (Fig. 12a) nor atropine (5×10^{-6}M) (Fig. 12b) significantly altered the inhibition by 2-chloroadenosine of nerve-mediated responses of rat vas deferens, the 2-substituted adenosine derivatives appeared not to act on these receptors.

Phenoxybenzamine per se did not effect the magnitude of the twitch response but abolished the secondary sustained responses of the rat vas deferens to electrical field stimulation.

EFFECT OF INDOMETHACIN ON INHIBITION BY 2-CHLOROADENOSINE

ATP and ADP stimulate the synthesis of prostaglandins and prostaglandins have been shown to inhibit noradrenergic transmission in the vas deferens presynaptically. Indomethacin (5×10^{-5}M for 60 min), at a concentration which is greater than the ID_{50} for inhibition of prostaglandin synthesis in many tissues, did not significantly alter the inhibition by 2-chloroadenosine of twitch or sustained responses of the rat vas deferens to electrical field stimulation (Fig. 12c). Therefore, the 2-substituted adenosine derivatives were probably not acting through the stimulation of prostaglandin synthesis.

INHIBITION OF ADENOSINE UPTAKE

(A) Effect of dipyridamole

Adenosine is taken up in many tissues and this uptake of adenosine may limit its potency at extracellular receptor sites. In the presence of dipyridamole (10^{-5}M), an inhibitor of adenosine uptake, the inhibition by adenosine of twitch and sustained responses of rat vas deferens to electrical field stimulation was potentiated. This is shown in

FIG. 12 Inhibition by 2-chloroadenosine of twitch (——) and sustained (---) responses of rat vas deferens to field stimulation (5 Hz, 30 s) in normal Krebs (■) and in the presence of phenoxybenzamine (5 μM; □ ; a) or atropine (5 μM; □ ; b) or of tissues pretreated with indomethacin (50 μM for 1 hr; □ ; c). Results are expressed as mean inhibition ± S.E.M., n=4.

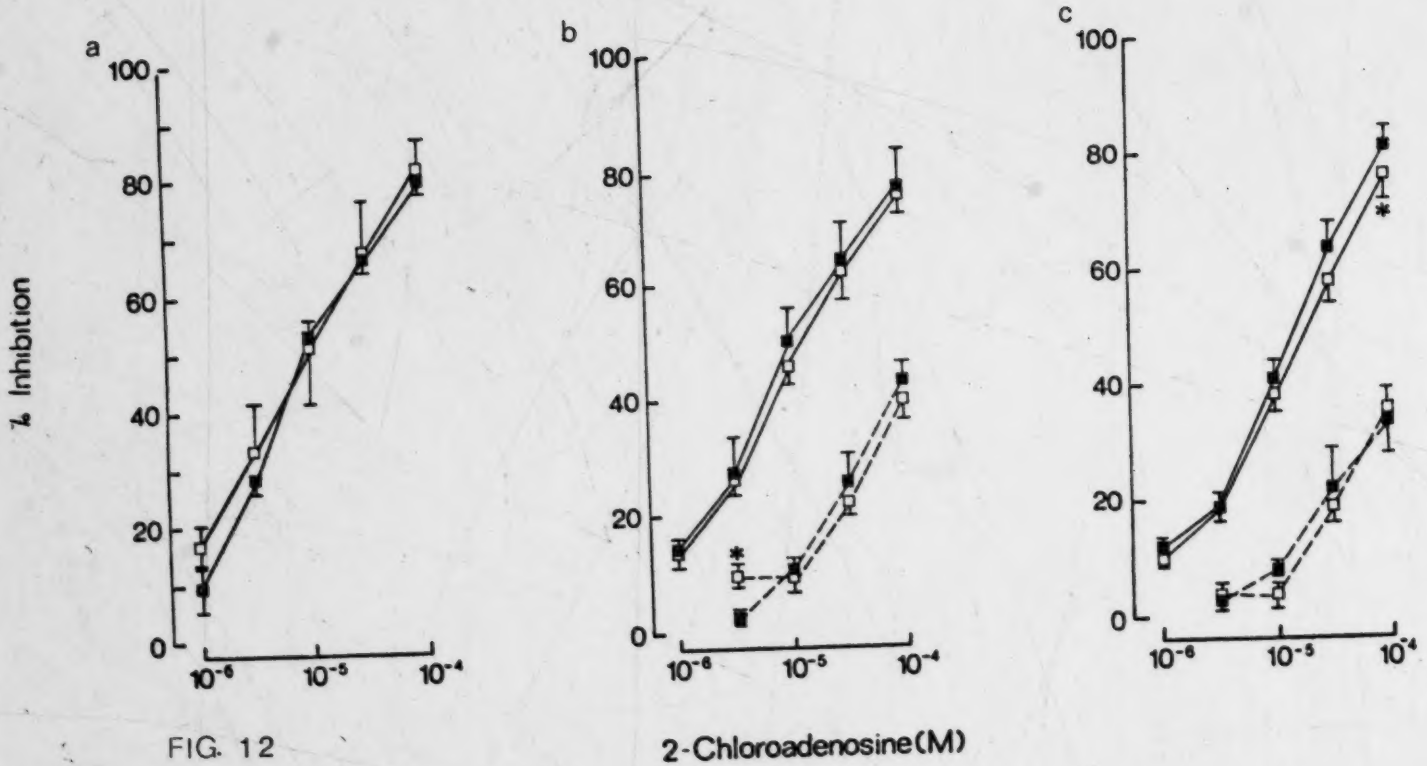
a
b
c
% Inhibition
100
80
60
40
20
0
10^{-6}
10^{-5}
10^{-4}
2-Chloroadenosine (M)

FIG. 12

Figure 13 a and b as a parallel shift to the left of the dose-response curve of adenosine and suggested that in this tissue the inhibitory action of adenosine is limited by its rapid uptake into cells. In contrast, the inhibition by 2-chloroadenosine of responses of this tissue to electrical field stimulation was not potentiated by dipyridamole (10^{-5}M); however, as shown in Figure 13 a and b, a trend towards antagonism of the inhibition by 2-chloroadenosine of sustained responses was apparent. These results suggested that uptake does not limit the inhibitory activity of 2-chloroadenosine.

The concentration of dipyridamole used in the present study probably did not have non-specific effects, since 10^{-5}M dipyridamole *per se* did not significantly alter the twitch or sustained frequency response curves of the rat vas deferens.

(B) Effect of HNBTG

The above results were confirmed and extended with another inhibitor of adenosine uptake. Pretreatment of rat vas deferens with HNBTG (10^{-5}M for 30 min) caused a potentiation of the inhibition by adenosine of responses to electrical field stimulation. This is shown in Figure 13 c and d, as a parallel shift to the left of the dose-response curves of adenosine. As with dipyridamole, the inhibition by 2-chloroadenosine of responses of this tissue to electrical field stimulation was not potentiated by HNBTG (10^{-5}M for 30 min). However, as shown in Figure 13 c and d, the inhibitory action of 2-chloroadenosine on sustained responses appeared to be antagonized by pretreatment of tissues with HNBTG.

Pretreatment of rat vas deferens with HNBTG (10^{-5}M for 30 min) did not alter the inhibition by 2-hydroxyadenosine or 2-bromoadenosine of

FIG. 13 Effect on twitch (a,c) and sustained (b,d) responses of rat vas deferens to field stimulation (5 Hz, 30 s) of adenosine (●) and 2-chloroadenosine (■) in normal Krebs, and of adenosine (○) and 2-chloroadenosine (□) in the presence of 10^{-5}M dipyridamole (a,b) or after pretreatment of tissues with HNBTG (10^{-5}M for 30 min) (c,d). Results are expressed as mean inhibition ± S.E.M., n=6. Asterisks represent the significance of the difference from the inhibition in normal Krebs of untreated tissues; * $0.05 > P > 0.01$; ** $0.01 > P > 0.001$; *** $P < 0.001$.

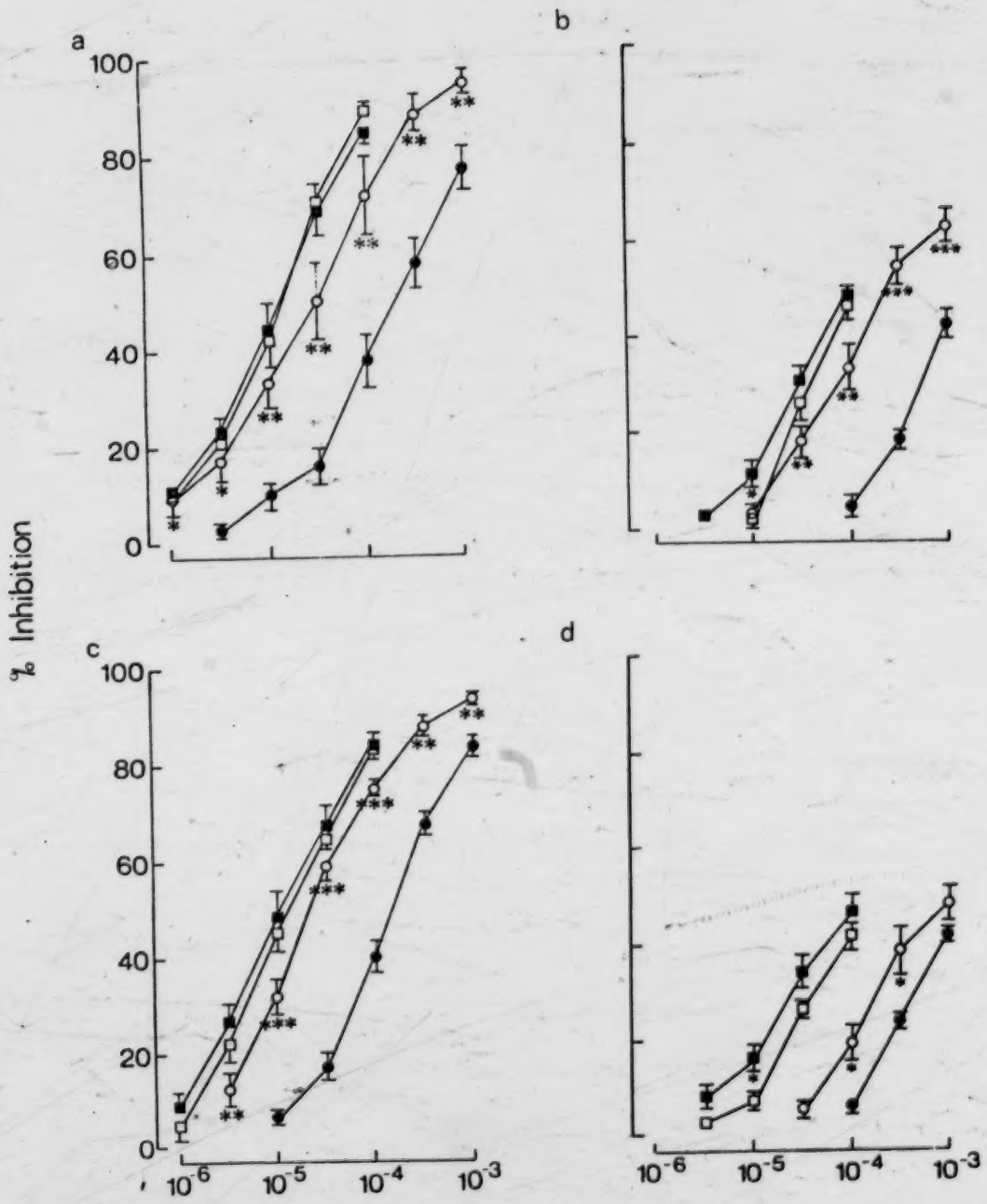
a
b
c
d
% Inhibition
100
80
60
40
20
0
10^{-6}
10^{-5}
10^{-4}
10^{-3}
Log Concentration (M)

FIG. 13

responses to electrical field stimulation. However, HNBTG caused a small but significant potentiation of the inhibitory action of 2-fluoroadenosine (Fig. 14 a, b and c). These studies with HNBTG suggest that 2-hydroxyadenosine and 2-bromoadenosine are not taken up into the vas deferens, however, 2-fluoroadenosine appears to be taken up into the tissue to some extent and this uptake is limiting to the inhibitory activity of 2-fluoroadenosine.

Pretreatment of rat vas deferens with 10^{-5}M HNBTG for 30 minutes did not significantly alter the frequency-response determinations for twitch and sustained responses. Therefore, pretreatment of tissues with the adenosine uptake inhibitor did not appear to result in non-specific depression or augmentation of nerve-mediated responses.

INHIBITION OF ADENOSINE DEAMINASE BY 2'-DEOXYCOFORMYCIN

2'-Deoxycoformycin, an inhibitor of adenosine deaminase, significantly potentiated the inhibition by adenosine of twitch and sustained responses of rat vas deferens to electrical field stimulation while having no effect on the inhibition by 2-chloroadenosine (Fig. 15 a and b). This suggests that adenosine is deaminated in this tissue and that deamination is limiting to its inhibitory activity. However, 2-chloroadenosine does not seem to be deaminated in the vas deferens. The potentiation of the effects of adenosine occurred only at adenosine concentrations above 10^{-5}M and resulted in a shift to the left of the adenosine dose-response curve. The potentiation of the inhibition by adenosine with 2'-deoxycoformycin (2×10^{-6}M for 30 min) was less than the potentiation caused by pretreatment of tissues with HNBTG (10^{-5}M for 30 min).

Pretreatment of tissues with 2×10^{-6}M 2'-deoxycoformycin for 30

FIG. 14 Inhibition by 2-hydroxyadenosine (a), 2-bromoadenosine (b) and 2-fluoroadenosine (c) of twitch (——) and sustained (---) responses of rat vas deferens to field stimulation (5 Hz, 30 s) in normal tissues (♦ ; ▼ , ▲) and in tissues pretreated with HNBTG (10^{-5}M for 30 min; ◊ , ▽ , △). Results are expressed as mean inhibition ± S.E.M., n=6. Asterisks represent the significance of the difference from normal tissues; * $0.05 > P > 0.01$; ** $0.01 > P > 0.001$; *** $P < 0.001$.

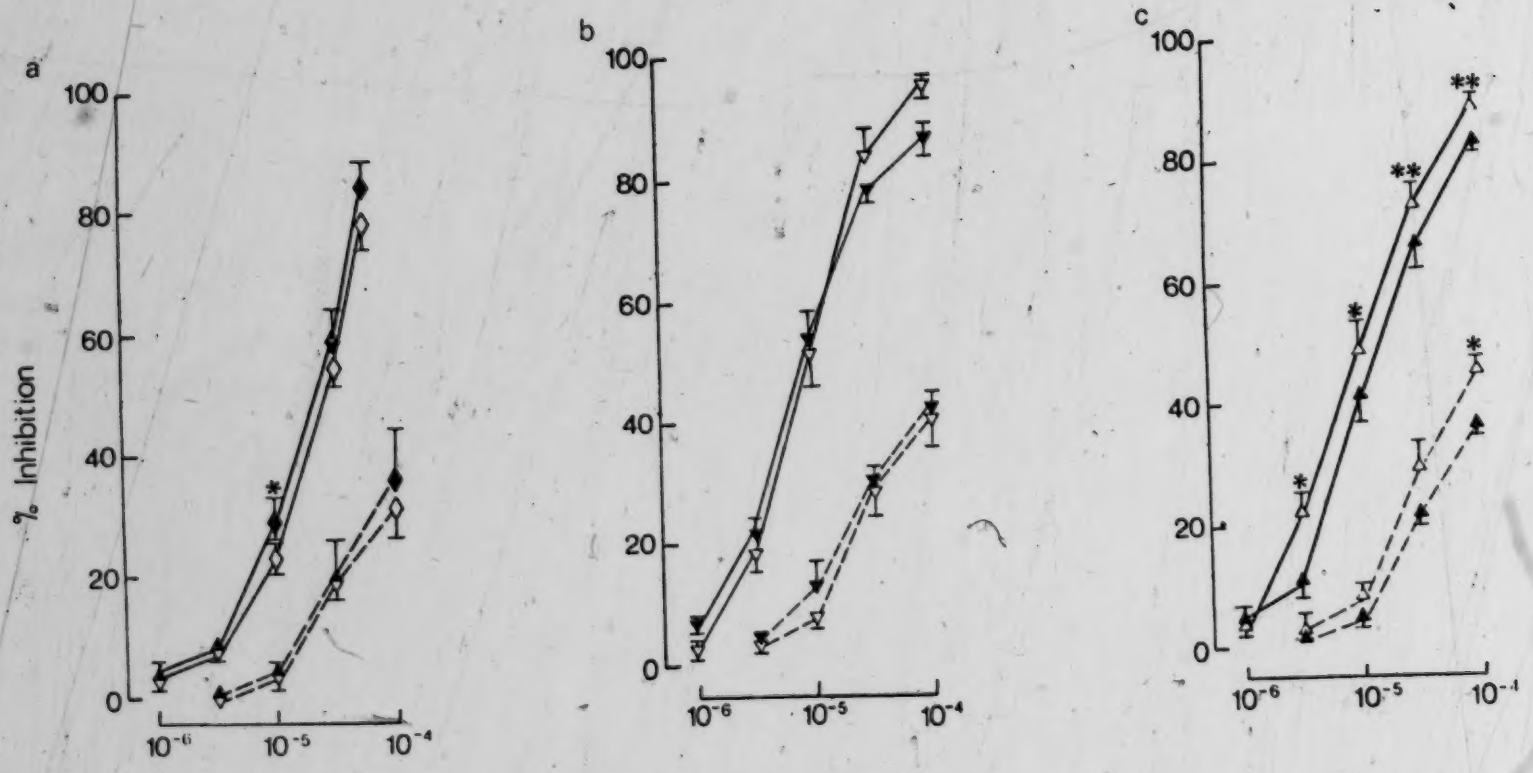
a
b
c
% Inhibition
100
80
60
40
20
0
10^{-6}
10^{-5}
10^{-4}
Log Concentration (M)

FIG. 14

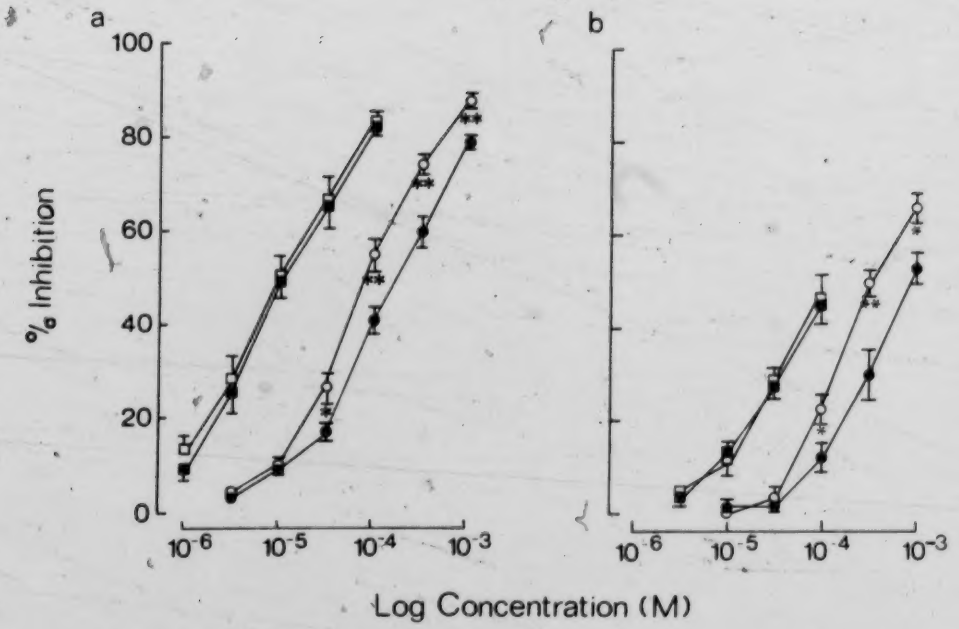

FIG. 15 Effect of twitch (a) and sustained (b) responses of rat vas deferens to field stimulation (5 Hz, 30 s) of adenosine (●) and 2-chloroadenosine (■) in normal Krebs and of tissues pretreated with 2'-deoxycoformycin (2×10^{-6}M for 30 min) (○; □). Results are expressed as mean inhibition ± S.E.M., n = 6. Asterisks represent the significance of the difference from untreated tissues; * $0.05 > P > 0.01$; ** $0.01 > P > 0.001$; *** $P < 0.001$.

minutes appeared to cause total inhibition of adenosine deaminase, since 10^{-5}M 2'-deoxycoformycin for 30 minutes did not cause a further potentiation of the effects of adenosine (Fig. 16).

The pretreatment of rat vas deferens with 2'-deoxycoformycin (2 x 10^{-6}M for 30 min) did not appear to result in non-specific depression or augmentation of responses, since the frequency-response determinations for twitch and sustained responses of this tissue were not significantly altered by pretreatment with 2'-deoxycoformycin per se.

COMBINATION OF ADENOSINE UPTAKE BLOCKADE AND DEAMINASE INHIBITION

There was no difference between dose-response curves of the inhibition by adenosine of responses of rat vas deferens to electrical field stimulation when tissues were pretreated with 2'-deoxycoformycin (2 x 10^{-6}M for 30 min) and then HNBTG (10^{-5}M for 30 min) or when tissues were pretreated with these drugs in the reverse order (Fig. 17). This suggests that 2'-deoxycoformycin does not utilize the adenosine uptake carrier to enter cells and that either pretreatment procedure can be used.

Pretreatment of the rat vas deferens with 2'-deoxycoformycin (2 x 10^{-6}M for 30 min) as well as with HNBTG (10^{-5}M for 30 min) caused a greater potentiation of the inhibitory action of adenosine on responses of tissues to electrical field stimulation than seen with pretreatment of tissues with only HNBTG (Fig. 18). Therefore, this suggests that both uptake and deamination of adenosine are limiting to the inhibitory activity of adenosine and that these limitations occur by different mechanisms.

The inhibitory potency on responses to electrical field stimulation

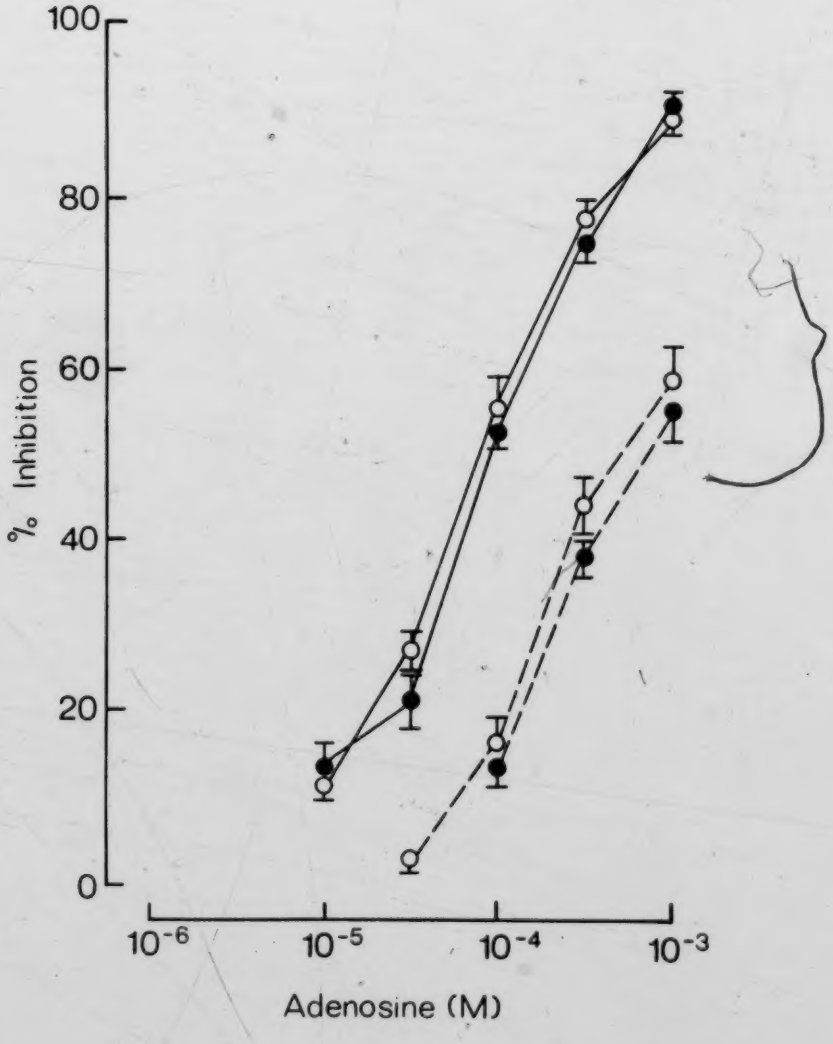

FIG. 16 Inhibition by adenosine of twitch (——) and sustained (- - -) responses of rat vas deferens to field stimulation (5 Hz, 30 s) in tissues pretreated with 2'-deoxycoformycin (2×10^{-6}M for 30 min) (●) or with 2'-deoxycoformycin (10^{-5}M for 30 min) (0). Results are expressed as mean inhibition ± S.E.M., n = 6.

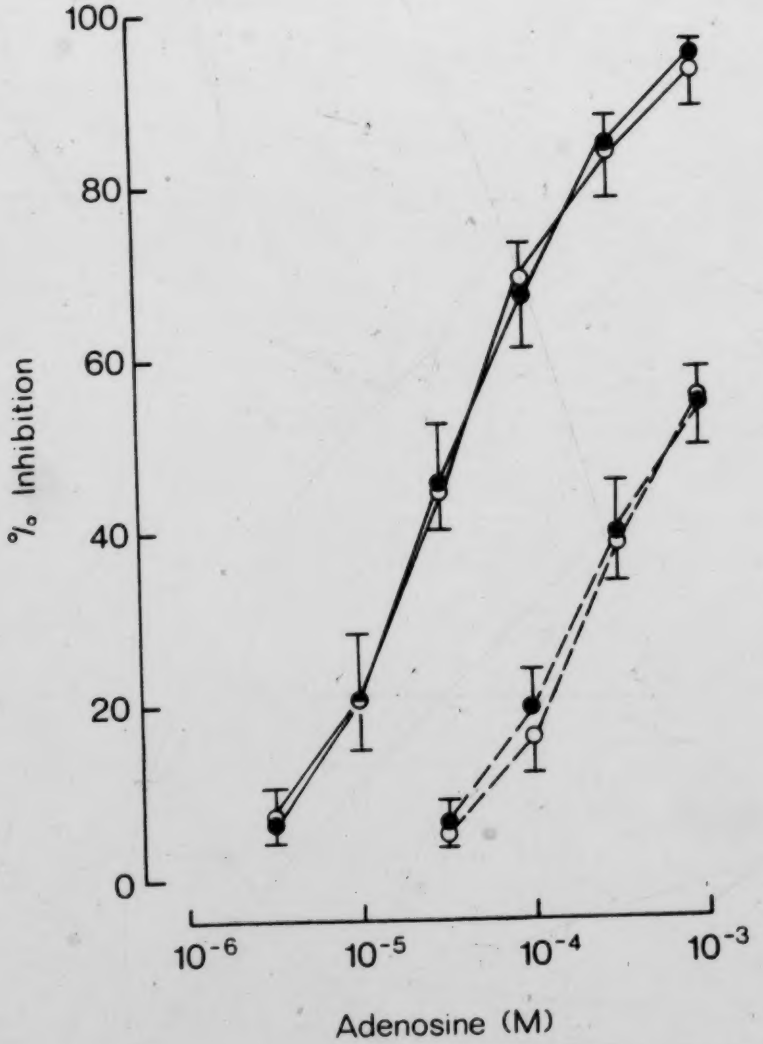

FIG. 17 . Inhibition by adenosine of twitch (——) and sustained (- - -) responses of rat vas deferens to field stimulation (5 Hz, 30 s) in tissues pretreated with 2'-deoxycoformycin (2×10^{-6}M for 30 min) then HNBTG (10^{-5}M for 30 min) (●), or in tissues pretreated with these drugs in the reverse order (0). Results are expressed as mean inhibition ± S.E.M., n = 4.

FIG. 18 Inhibition by adenosine of twitch (——) and sustained (---) responses of rat vas deferens to field stimulation (5 Hz, 30 s) in tissues pretreated with HNBTG (10^{-5}M for 30 min) (●), or in tissues pretreated with both HNBTG (10^{-5} M for 30 min) and 2'-deoxycoformycin (2 x 10^{-6}M for 30 min) (O). Results are expressed as mean inhibition ± S.E.M., n=6. Asterisks represent the significance of the difference from tissues pretreated with only HNBTG; * $0.05 > P > 0.01$; ** $0.01 > P > 0.001$; ***$P < 0.001$.

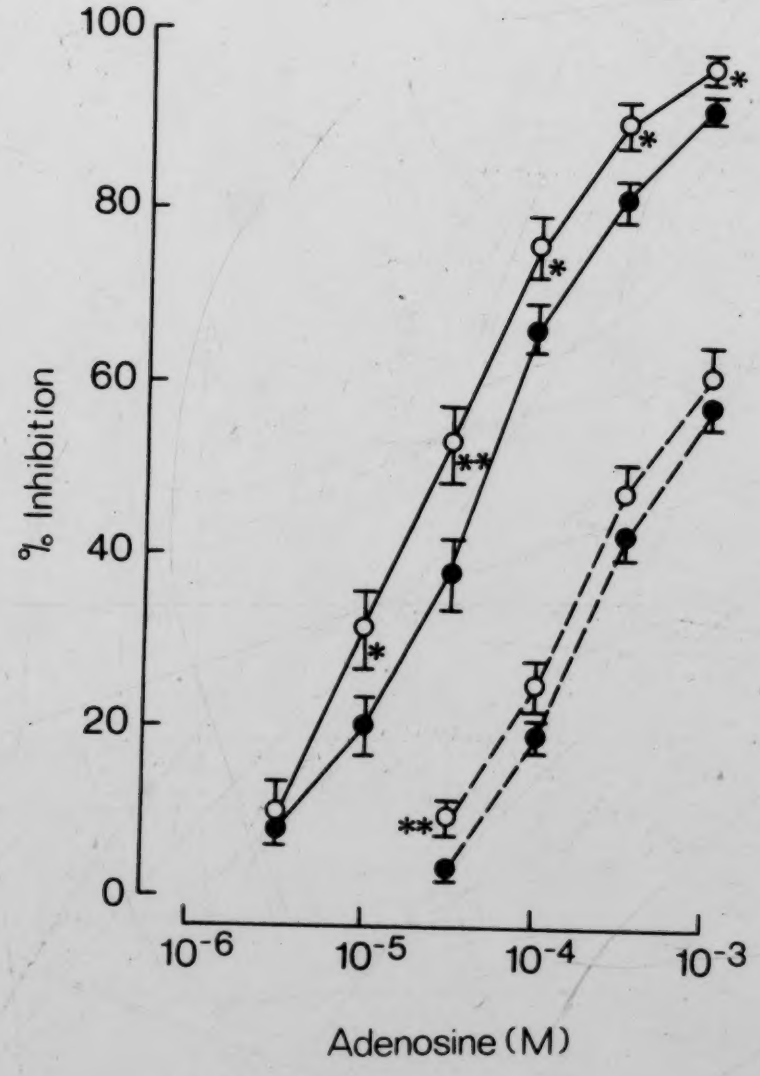

FIG. 18

of 2-chloroadenosine was not significantly different from that of adenosine when the rat vas deferens was pretreated with both 2'-deoxycoformycin (2×10^{-6}M for 30 min) and HNBTG (10^{-5}M for 30 min) (Fig. 19). Therefore, 2-chloroadenosine and adenosine appear to be equally potent at the receptor level.

Pretreatment of rat vas deferens with both HNBTG (10^{-5}M for 30 min) and 2'-deoxycoformycin (2×10^{-6}M for 30 min) per se did not significantly alter the frequency-response curves of the twitch or sustained responses of this tissue to electrical field stimulation (Fig. 20). Therefore, a physiological significance for adenosine on these responses in this tissue is questionable.

DURATION OF ACTION OF ADENOSINE AND THE 2-SUBSTITUTED ADENOSINE DERIVATIVES

Adenosine and 2-aminoadenosine were short acting, with responses returning to normal within the five minute time cycle of the experiments after washing. 2-Fluoroadenosine was slightly longer acting and inhibited responses of rat vas deferens for at least 5 minutes following the beginning of washout of drug by overflow. The other 2-substituted adenosine derivatives (2-chloroadenosine, 2-hydroxyadenosine and 2-bromoadenosine) caused a prolonged inhibition of nerve-mediated responses of tissues. Reproducible responses similar to the initial controls were obtained about 30 minutes following administration of the ID_{50} dose of these compounds and subsequent washout by overflow, furthermore, higher concentrations of these 2-substituted adenosine derivatives required longer periods of washout. Washout times were not limited by washout of drug from the organ bath, since increasing the flow of Krebs solution

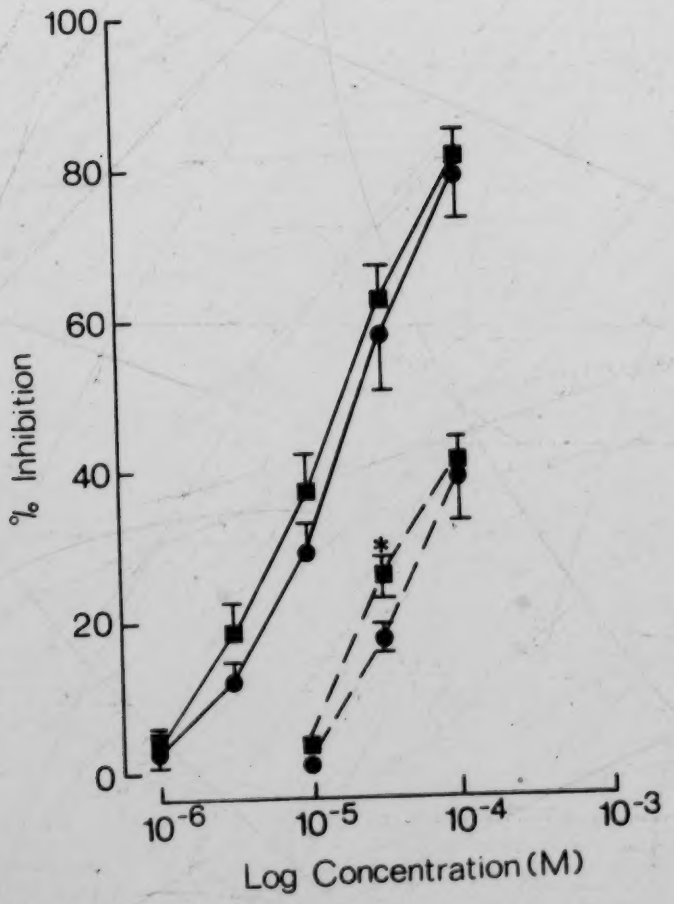

FIG. 19 Inhibition by adenosine (●) and 2-chloroadenosine (■) of twitch (——) and sustained (- - -) responses of rat vas deferens to field stimulation (5 Hz, 30 s) in tissues pre-treated with HNBTG (10^{-5}M for 30 min) and 2'-deoxycoformycin (2×10^{-6}M for 30 min). Results are expressed as mean inhibition ± S.E.M., n = 6.

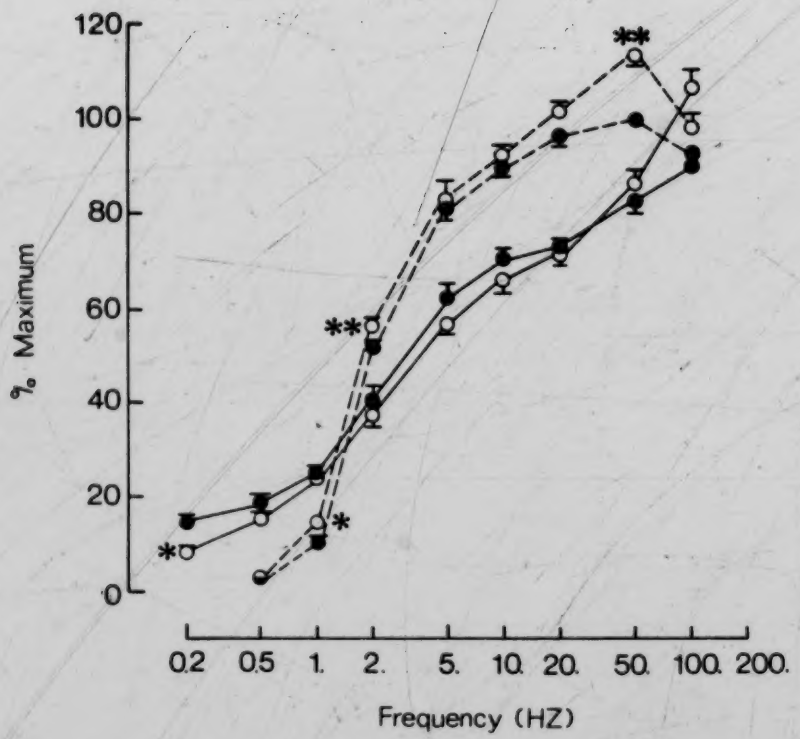

FIG. 20 Plot of frequency-response curves of twitch (———) and sustained (- - -) responses of middle portions of rat vas deferens to field stimulation in normal tissues (●) and in tissues pretreated with both HNBTG (10^{-5}M for 30 min) and 2'-deoxycoformycin (2 x 10^{-6}M for 30 min) (0). Results are expressed as mean % maximum ± S.E.M., n = 4. Asterisks represent the significance of the difference from normal tissues; * $0.05 > P > 0.01$; ** $0.01 > P > 0.001$; *** $P < 0.001$.

did not decrease the time required to obtain reproducible contractions.

The difference in time required to obtain reproducible contractions following adenosine and 2-chloroadenosine administration was not eliminated by pretreatment of tissues with HNBTG and 2'-deoxycoformycin. However, the duration of action of adenosine was prolonged to the equivalent to that of 2-fluoroadenosine by pretreatment of rat vas deferens with these inhibitors of adenosine uptake and deamination.

EFFECT OF THEOPHYLLINE ON INHIBITION BY ADENOSINE

Theophylline (10^{-4}M) appeared to antagonize the inhibition by adenosine of twitch and sustained responses of rat vas deferens to electrical field stimulation (Fig. 21). However, contrary to similar experiments with 2-substituted adenosine derivatives, this antagonism by theophylline was not significant. But when tissues were pretreated with both 2'-deoxycoformycin (2×10^{-6}M for 30 min) and HNBTG (10^{-5}M for 30 min), drugs which shift the adenosine dose-response curve to the left, the antagonism by theophylline of the inhibitory action of adenosine on twitch and sustained responses to electrical field stimulation was significant. This is shown in Figure 22 as a parallel shift to the right of the dose-response curves of adenosine. Therefore, although there was a trend towards antagonism by theophylline of the inhibitory action of adenosine, this antagonism only became significant when both uptake and deamination of adenosine were inhibited.

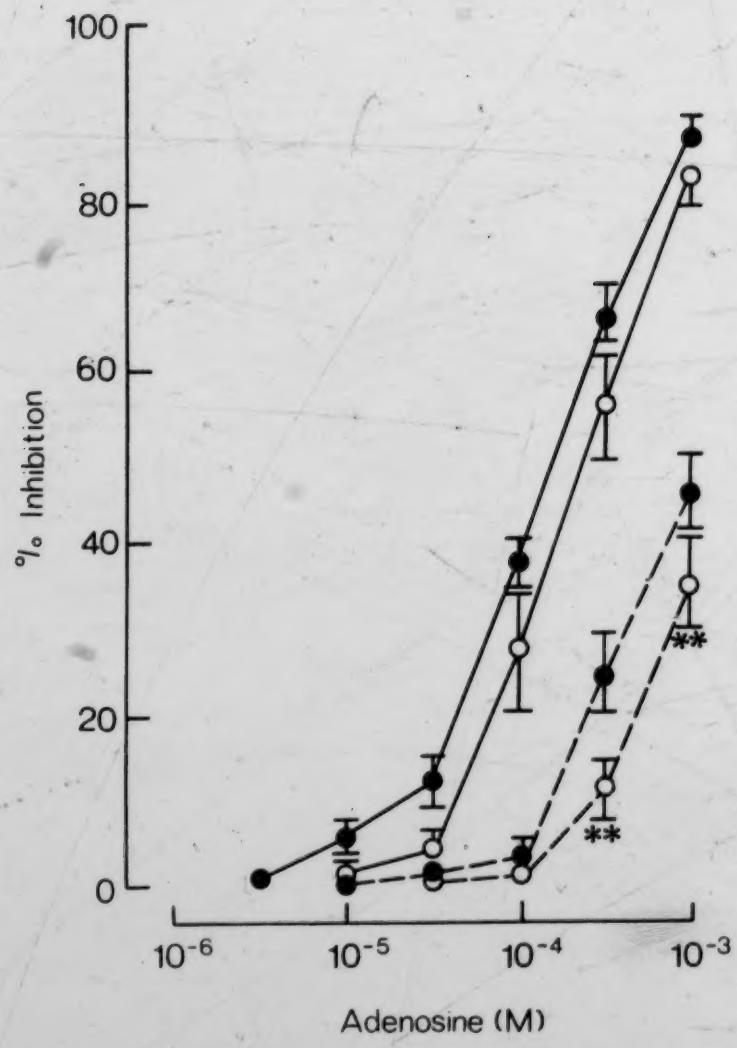

FIG. 21 Inhibition by adenosine of twitch (——) and sustained (---) responses of rat vas deferens to field stimulation (5 Hz, 30 s) in normal Krebs (●) and in the presence of theophylline (10^{-4}M for 30 min) (○). Results are expressed as mean inhibition ± S.E.M., n=6. Asterisks represent the significance of the difference from Krebs; * 0.05 > P > 0.01; ** 0.01 > P > 0.001; *** P < 0.001.

FIG. 22 Inhibition by adenosine of twitch (——) and sustained (---) responses of rat vas deferens to field stimulation (5 Hz, 30 s) of tissues pretreated with HNBTG (10^{-5}M for 30 min) in normal Krebs (●) or in the presence of theophylline (10^{-4}M for 30 min) (○). Results are expressed as mean inhibition ± S.E.M., n=6. Asterisks represent the significance of the difference from normal Krebs: * $0.05 > P > 0.01$; ** $0.01 > P > 0.001$; *** $P < 0.001$.

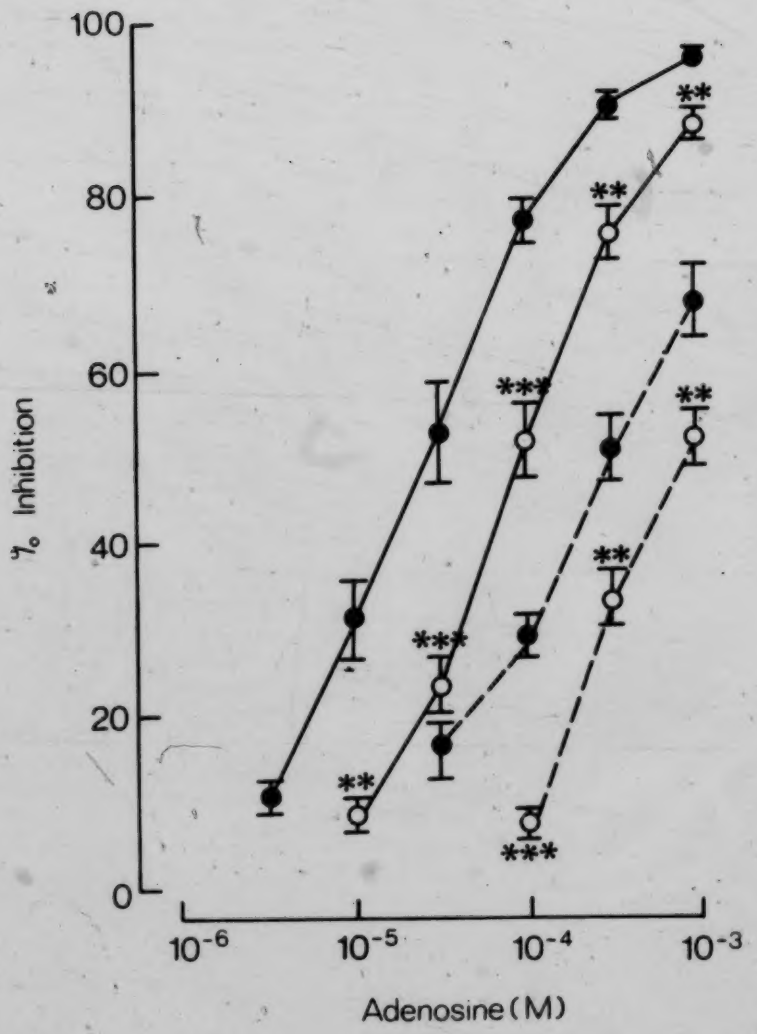

FIG. 22

DISCUSSION

STATEMENT OF THE PROBLEM

An aim of the present investigation was to compare the effects of 2-substituted adenosine derivatives with those of adenosine on noradrenergic transmission in the rat vas deferens in vitro, a tissue which possesses presynaptic adenosine receptors (Clanachan et al., 1977). The study began with the observation in this laboratory, subsequent to a structure-activity study with adenosine derivatives on presynaptic inhibition by Paton et al. (1978), that 2-chloroadenosine was more potent and longer acting than adenosine in inhibiting responses of this tissue due to electrical field stimulation. Although these observations have been made in many adenosine sensitive systems (Clarke et al., 1952; Mihich et al., 1954; Born, 1964; Constantine, 1965; Michal and Thorp, 1966; Gough et al., 1969; Angus et al., 1971; Gilman, 1974; Huang and Daly, 1974; Blume and Foster, 1975; Phillis and Kostopoulos, 1975; Satchell and Maguire, 1975; Huang and Drummond, 1976), there was no adequate explanation for the high apparent potency of 2-chloroadenosine compared to adenosine. Therefore, the major question was as follows: Why does 2-substitution of adenosine result in a drug which is more potent than the parent compound?

RESPONSES OF RAT VAS DEFERENS TO ELECTRICAL FIELD STIMULATION

Electrical field stimulation of the rat vas deferens elicits a rapid initial twitch response lasting about five seconds, immediately followed by a slower secondary sustained contraction (cf. Swedin, 1971; Clanachan et al., 1977). Further, field stimulation of the urethral portion of this tissue results mainly in twitch responses while sustained responses are mainly elicited from the testicular portion

(Duncan and McGrath, 1976). These observations have been confirmed in the present investigation and indicated that in order to study the effects of drugs on both twitch and sustained responses, the middle portion of the vas deferens must be utilized which consisted of both urethral and testicular portions of the tissue.

An unusual observation arose from using both urethral and testicular portions of the rat vas deferens. The frequency-response curves of the twitch and sustained responses appeared biphasic in shape, with a second increase in slope occurring at frequencies above 20 Hz. Direct muscle stimulation could not explain the secondary rise in these curves, since TTX, a blocker of action potentials in neurones, eliminated all responses of the tissue to field stimulation. Therefore, the responses were nerve-mediated. There are two other possible explanations for the secondary increase in the frequency-response curves. Two sets of fibers may innervate the tissue, with one set of fibers firing preferentially at low frequencies of stimulation while the other set of fibers may fire preferentially at high frequencies of stimulation. Alternatively, an interaction between two somewhat different portions which are joined to make one tissue may explain this biphasic phenomenon. Therefore, frequency-response curves were determined for the twitch response from the urethral portion of the rat vas deferens and for the sustained response from the testicular portion of this tissue. The frequency-response curve for the twitch response was linear with a maximum occurring at 100 Hz. On the other hand, an S-shaped curve of sustained responses with a maximum occurring at 20 Hz resulted from the testicular portion of the vas deferens. These results suggest that the biphasic shape of the frequency-response curves from the middle portion of the

rat vas deferens may be due to a physical interaction of the urethral and testicular portions of this tissue, however, the possibility of dual innervation has not been excluded. Furthermore, these results indicate that although the biphasic shape of the frequency-response curves from the middle portion of the vas deferens are atypical, this should not limit the use of both portions of the tissue at once. Therefore, the effects of drugs on the twitch and sustained responses of rat vas deferens were studied simultaneously in the present investigation.

There were two reasons for studying effects of drugs on both twitch and sustained responses of rat vas deferens to electrical field stimulation. First, twitch responses have been shown to be more sensitive than sustained responses to the inhibitory actions of adenosine (Clanachan et al., 1977); and second, both of these responses may be due to noradrenaline. Although there is no doubt that sustained responses of vas deferens are noradrenergic in nature (cf. Swedin, 1971), there has been some concern as to the nature of the twitch response of the tissue to electrical field stimulation (Ambache and Zar, 1971; Euler and Hedqvist, 1975; Simon and Van Maanen, 1976). However, the bulk of the evidence suggests that noradrenaline does mediate twitch responses as well as sustained responses (cf. Swedin, 1971; Furness, 1971; Furness and Iwayama, 1972; Furness, 1974; Jones and Spriggs, 1975). Therefore, if a drug alters responses due to nerve stimulation differently than responses due to exogenous noradrenaline which acts solely postsynaptically, then the drug probably has a presynaptic site of action.

PRESYNAPTIC INHIBITION BY ADENOSINE AND 2-SUBSTITUTED ADENOSINE DERIVATIVES

Adenosine and the 2-substituted adenosine derivatives (2-chloroadenosine, 2-hydroxyadenosine, 2-bromoadenosine, 2-fluoroadenosine and 2-aminoadenosine) inhibited both submaximal twitch and sustained responses of the rat vas deferens to electrical field stimulation. However, these compounds had very much less inhibitory activity on submaximal responses to exogenous noradrenaline. Therefore, the 2-substituted adenosine derivatives appear to have a predominately presynaptic site of action as has been shown for adenosine (cf. Clanachan et al., 1977). Adenosine and the adenine nucleotides have been shown to inhibit responses of the rat vas deferens by decreasing the amount of [^{3}H]-noradrenaline released by nerve stimulation. Probably the 2-substituted adenosine derivatives also inhibit noradrenaline release due to nerve stimulation and thereby decrease responses to electrical field stimulation; however, this has not been demonstrated by monitoring [^{3}H]-noradrenaline release in the present study. At high concentrations, adenosine and the 2-substituted adenosine derivatives inhibited responses due to exogenous noradrenaline; therefore, these compounds also have some postsynaptic action. This was not reported for adenosine in the earlier study by Clanachan et al. (1977).

DIFFERENTIAL SENSITIVITIES OF TWITCH AND SUSTAINED RESPONSES

Twitch responses of the rat vas deferens were significantly more inhibited than sustained responses by adenosine and the 2-substituted adenosine derivatives. This observation has also been made by Clanachan et al. (1977), but no explanation was forwarded. One

explanation for this differential sensitivity may be derived from morphological considerations in this tissue. The vas deferens is very densely innervated with each smooth muscle cell receiving individual innervation (Richardson, 1962). Nerve terminals synapse in shallow depressions or may even be enclosed with smooth muscle cells (Furness and Iwayama, 1971). The neuromuscular junctions of the rat vas deferens are narrow (about 200 Å) (Richardson, 1962), thus implying a high efficiency of transmission and uptake of transmitter (cf. Holman, 1970; Swedin, 1971; Furness and Iwayama, 1971). Swedin (1971) has suggested an explanation for the twitch and sustained responses of the vas deferens to electrical field stimulation based on the morphology of innervation of this tissue. The suggestion was that a rapid release of noradrenaline which would then stimulate alpha-adrenoceptors located in a narrow synapse, together with efficient noradrenaline uptake, forms the physiological basis of the twitch response. The sustained responses would occur after sufficient noradrenaline spilled out of the neuromuscular junction to stimulate extrajunctional alpha-adrenoceptors. There appears to be no difference between junctional and extrajunctional alpha-adrenoceptors other than location (Hotta, 1969). Therefore, differential sensitivity of twitch and sustained responses to adenosine and related compounds may result from a finer control of the concentration of noradrenaline in a narrow synapse than on extrajunctional sites.

ADENOSINE RECEPTOR-MEDIATED INHIBITION

Theophylline, a commonly used postsynaptic adenosine receptor antagonist (cf. Burnstock, 1975), antagonized the inhibition by the 2-substituted adenosine derivatives of twitch and sustained responses of

the rat vas deferens to electrical field stimulation. This inhibition was expressed as a parallel shift to the right of the dose-response curves of these compounds. Only one dose of theophylline was tested and the maximum inhibition by the 2-substituted adenosine derivatives was not achieved in the presence of antagonist; therefore, no conclusions can be drawn with respect to whether the antagonism by theophylline was competitive. These experiments suggest that in the rat vas deferens the 2-substituted adenosine derivatives, like adenosine, probably act via a purinergic receptor to inhibit noradrenergic transmission. However, from these experiments nothing can be said about the specificity of action of the 2-substituted adenosine derivatives.

Alpha-agonist (Vizi et al., 1973) and muscarinic-agonists (Stjarne, 1975; Leighton and Westfall, 1976) have been shown to inhibit noradrenergic transmission in the vas deferens and these presynaptic inhibitors may be of physiological importance in this tissue (cf. Starke, 1977; Westfall, 1977). Therefore, an interaction of the 2-substituted adenosine derivatives with the alpha-adrenoceptor or muscarinic-receptor was possible. Furthermore, since adenine nucleotides have been shown to stimulate the synthesis of prostaglandins (Needleman et al., 1974), and prostaglandins in turn inhibit noradrenergic transmission in the vas deferens (Hedqvist, 1974), the 2-substituted adenosine derivatives may have acted partially via a prostaglandin mechanism. Phenoxybenzamine, atropine or indomethacin did not alter the inhibition of responses of rat vas deferens to nerve stimulation by 2-chloroadenosine, suggesting no interaction of 2-chloroadenosine with the above-mentioned mechanism. Therefore, in this tissue, the 2-substituted adenosine derivatives probably decrease noradrenaline release by nerve stimulation specifically

by an action on the same presynaptic receptor system as adenosine.

POTENCY OF ADENOSINE AND DERIVATIVES

Some of the 2-substituted adenosine derivatives were more potent and longer acting than adenosine in inhibiting responses of the rat vas deferens to electrical field stimulation. 2-Chloroadenosine was the most potent and longest acting. 2-Hydroxyadenosine and 2-bromoadenosine were almost as potent and long acting as 2-chloroadenosine. Although 2-fluoroadenosine was more potent than adenosine, it was relatively short acting. 2-Substitution *per se* was not sufficient to result in a compound which was more potent and longer acting than adenosine, since 2-aminoadenosine had the same potency and duration of action as adenosine on nerve-mediated responses in this tissue. Results obtained in the rat vas deferens are in agreement with observations by other investigators. A high apparent potency and long duration of action of various 2-substituted adenosine derivatives have been demonstrated in numerous systems including other smooth muscles: For example, blood vessels (Clarke *et al.*, 1952; Gough *et al.*, 1969; Angus *et al.*, 1971; Cobbin *et al.*, 1974) and guinea-pig taenia coli (Satchell and Maguire, 1975). 2-Fluoroadenosine has also been shown to be more potent than adenosine in relaxing smooth muscle (Cobbin *et al.*, 1974) and this compound was also more potent than adenosine in inhibiting nerve-mediated responses of vas deferens. On the other hand, 2-fluoroadenosine does not activate adenylate cyclase (Huang and Drummond, 1978). These results suggest that structural requirements are similar for pre- and postsynaptic adenosine activity in smooth muscle, but differences exist between these structural requirements and those necessary for stimulation

of adenylate cyclase.

Although 2-chloroadenosine has been found to be more potent than adenosine in many systems, this observation has not been explained adequately. Several actions of 2-chloroadenosine could conceivably result in a compound with a high apparent potency: For example, 2-chloroadenosine has been shown to inhibit adenosine uptake in guinea-pig brain and ventricular slices (Huang and Daly, 1974; Huang and Drummond, 1976) as well as to inhibit adenosine deaminase (Rockwell and Maguire, 1966; Maguire and Sim, 1971) and cyclic AMP phosphodiesterase (Wilkening and Makman, 1975). Rockwell and Maguire (1966) found that there was no correlation between potency of the compound and its inhibitory activity on adenosine deaminase. When comparing the actions of 2-chloroadenosine and dipyridamole, Angus et al. (1971) concluded that these two compounds did not act by the same mechanism. Wilkening and Makman (1975) found that the cyclic AMP phosphodiesterase inhibitory property of 2-chloroadenosine could not explain its effect of increasing cyclic AMP levels. Therefore, these properties of 2-chloroadenosine cannot explain its high apparent potency compared to that of adenosine.

Previous work from this laboratory suggested that a carrier-mediated uptake process for adenosine exists in rat vas deferens (Clanachan et al., 1977). The present study is in agreement with this suggestion, dipyridamole and HNBTG potentiated the inhibition by adenosine of twitch and sustained responses of the rat vas deferens to electrical field stimulation. Dipyridamole has been shown to inhibit nucleoside transport in various cells (Buang et al., 1964; Scholtissek, 1968; Plagemann, 1971; Roos and Pfleger, 1972; Schrader et al., 1972; Kolassa et al., 1970; Olsson et al., 1972; Hopkins, 1973; Stachell et al., 1972), however,

dipyridamole has also been shown to possess a variety of non-specific actions (Bunag et al., 1964; Sneft, 1968; Plagemann and Roth, 1969; Renner et al., 1972; Himari and Taira, 1976). On the other hand, HNBTG may be quite specific in its action as an inhibitor of nucleoside transport (cf. Baer et al., 1978). Since results obtained with these two inhibitors of adenosine uptake were identical, the potentiation of the inhibitory actions of adenosine indeed appeared to be due to an inhibition of its uptake. These results indicate that the potency of adenosine is normally limited by the uptake process in rat vas deferens.

Contrary to these results, the inhibitory actions of 2-chloroadenosine, 2-hydroxyadenosine and 2-bromoadenosine on responses of the rat vas deferens to electrical field stimulation were not altered by HNBTG. Furthermore, dipyridamole did not alter the inhibitory action of 2-chloroadenosine in the present study. However, HNBTG did cause a small potentiation in the inhibitory activity of 2-fluoroadenosine. The compounds which were not potentiated by inhibitors of adenosine uptake are probably not taken up by the adenosine carrier in this tissue and therefore, the potency of these compounds is not limited by the uptake process via the adenosine carrier. It is unlikely that 2-chloroadenosine utilizes another efficient uptake system.

Previous reports with respect to the uptake of 2-chloroadenosine have been controversial. In the guinea-pig brain slice preparation, 2-chloroadenosine may be taken up by an adenosine uptake process, since, hexobendine potentiated the accumulation of cyclic AMP caused by 2-chloroadenosine (Huang and Daly, 1974). Cobbin et al. (1974), when studying the coronary vasodilator effects of 2-substituted adenosine derivatives in dogs in vivo, have suggested that adenosine derivatives with substitutions larger than fluorine in the 2-position may not be

accepted by an adenosine carrier. These authors found that the action of adenosine was potentiated by various adenosine derivatives and concluded that structural modification which resulted in an increased dilator potency was different from that of clearance from the vascular system. Furthermore, it was implied that the high apparent potency of these compounds was due to an enhanced activity at a vascular smooth muscle receptor (Cobbin et al., 1974). Therefore, the results and conclusions of the present study differ from those of both groups. In the rat vas deferens, the inhibitory potency of adenosine appears to be limited by uptake; however, the potency of 2-chloroadenosine and some other 2-substituted adenosine derivatives does not appear to be limited by uptake, and this partially explains the differences in inhibitory potency between adenosine and 2-chloroadenosine.

The observation that adenosine but not 2-chloroadenosine was taken up by the rat vas deferens and that adenosine uptake limited its inhibitory activity does not completely explain the difference in potency of these compounds. It has recently been suggested that metabolism of adenosine, subsequent to uptake, may limit its potency (cf. Satchell and Burnstock, 1975). Generally, metabolism of adenosine results in either phosphorylated or deaminated products. Although at this time there are no specific inhibitors of adenosine kinases, recently effective inhibitors of mammalian adenosine deaminase are available. Therefore, the question of limitation of adenosine potency by deamination could be tested experimentally.

Pretreatment of the rat vas deferens with 2'-deoxycoformycin, a tightly binding inhibitor of adenosine deaminase (Woo et al., 1974; Cha et al., 1975), resulted in a potentiation of the inhibitory activity

of adenosine. This suggests that adenosine is deaminated in the vas deferens limiting its inhibitory activity. Other investigators (Cass and AuYeung, 1976; Johns and Adamson, 1976; LePage et al., 1976) have shown that the cytotoxic effects and antitumor activity of several adenosine analogs which are metabolized by adenosine deamination are increased by 2'-deoxycoformycin. The present study is the first demonstration of a potentiation of the effects of adenosine by inhibition of adenosine deaminase with 2'-deoxycoformycin.

Contrary to these results, the inhibitory activity of 2-chloroadenosine on responses of the rat vas deferens to electrical field stimulation was not altered by pretreatment of tissues with 2'-deoxycoformycin. Thus 2'-deoxycoformycin showed a specific potentiating effect on adenosine action, 2-chloroadenosine not being metabolised by adenosine deaminase as has been established with mammalian deaminases generally (Clarke et al., 1952; Rockwell and Maguire, 1966; Maguire and Sim, 1971). The possibility that 2-chloroadenosine is degraded by adenosine deaminase in vas deferens and that the inhibitory action of 2-chloroadenosine was not potentiated by 2'-deoxycoformycin because the former is not taken up into this tissue is unlikely but has not been excluded.

To this point, both uptake and deamination separately have been shown to limit the inhibitory activity of adenosine in rat vas deferens. Taube and Berlin (1972) have suggested that adenosine entry and deamination are independent. We have found the following in the rat vas deferens. First, pretreatment of rat vas deferens with both HNBTG and 2'-deoxycoformycin resulted in a greater potentiation of the inhibitory activity of adenosine than pretreatment of tissues with either uptake

inhibitor or adenosine deaminase inhibitor alone. Second, the order of pretreatment with the two drugs was not a determinant of their combined effect. Since these processes limit the inhibitory activity of adenosine independently, these results support the suggestion of Taube and Berlin (1972) that uptake and deamination of adenosine are independent.

The combined potentiation of adenosine action in the rat vas deferens by simultaneous inhibition of uptake and deamination virtually eliminated the difference in the inhibitory potency between adenosine and 2-chloroadenosine. Therefore, it appears that adenosine and 2-chloroadenosine are, in fact, equipotent at the receptor level and the apparent difference in potency in untreated tissues appears to be a result of adenosine uptake and metabolism. Although our experiments involving both inhibition of uptake and deamination of adenosine produced dose-response curves for adenosine statistically not different from those for 2-chloroadenosine, the dose-response curves were never superimposable but always showed a small difference of about 0.1 to 0.2 log units. This may be real but very small, and thus difficult to measure quantitatively, difference of potencies for the two drugs at the receptor level. Alternatively, this small difference may be due to incomplete inhibition of adenosine uptake, or deamination, to selective action of adenosine kinase or to differences in passive diffusion of adenosine vs 2-chloroadenosine or combinations of such effects.

DURATION OF ACTION OF ADENOSINE AND DERIVATIVES

While the above experiments showed striking effects of HNBTG and 2'-deoxycoformycin on the potency of adenosine, the difference in the duration of action between adenosine and 2-chloroadenosine in the rat

vas deferens was not eliminated by inhibition of uptake and deamination of adenosine. In vivo studies of cardiovascular effects of adenosine and 2-chloroadenosine suggested that the long duration of action of 2-substituted adenosine derivatives is a result of resistance to enzymatic degradation (Clarke et al., 1952) or a result of not being taken up (Cobbin et al., 1974). In our experiments in the rat vas deferens in vitro, where the drug can be washed from the system, it appears that the duration of action of 2-chloroadenosine cannot be explained by either of these suggestions. Although there is no experimental evidence, 2-chloroadenosine may bind more tightly to the receptor or to other sites and thereby be harder to wash out of the tissue than adenosine.

ANTAGONISM BY THEOPHYLLINE OF ADENOSINE ACTION

Antagonism of 2-substituted adenosine derivatives inhibitory activity by theophylline in rat vas deferens has already been discussed, but alteration of adenosine effects by theophylline was not mentioned at that time. Theophylline has been shown to antagonize the effects of adenosine in many systems including the rat vas deferens (Clanachan et al., 1977). Contrary to this finding, although a trend was apparent in the present study, the antagonism by theophylline of the inhibitory activity of adenosine on nerve-mediated responses in rat vas deferens was not significant. However, when rat vasa were pretreated with inhibitors of adenosine uptake and deamination, using HNBTG and 2'-deoxycoformycin respectively, antagonism by theophylline was significant. There are several possible explanations for the increase in theophylline effectiveness when tissues were pretreated with inhibitors of adenosine uptake and deamination. Two possible explanations are as follows:

(1) HNBTG could potentiate theophylline-induced antagonism by decreasing the uptake of theophylline into cells;

(2) Pretreatment of tissues with these drugs could directly or indirectly alter adenosine receptors, and thus increase receptor affinity for theophylline.

Although the reason for the increased effectiveness of theophylline in rat vas deferens pretreated with inhibitors of adenosine uptake and deamination was not further investigated in the present study, this observation suggests that uptake and/or deamination processes may obscure true antagonist effectiveness. If so, these processes should be eliminated when attempting to classify adenosine receptors in different test systems. In this regard, cocaine has been shown to potentiate the effectiveness of alpha-adrenoceptor antagonists and of beta-adrenoceptor antagonists (cf. Furchgott, 1972).

PHYSIOLOGICAL SIGNIFICANCE OF PRESYNAPTIC ADENOSINE RECEPTORS

Pretreatment of rat vas deferens with HNBTG and 2'-deoxycoformycin did not significantly alter frequency-response curves suggesting that the inhibition of noradrenergic transmission by adenosine may not normally be of physiological significance in this tissue. If adenosine did play a physiological role in this regard, pretreatment of rat vas deferens with these inhibitors of adenosine uptake and deamination should effectively increase the endogenous concentration of purine in the neuromuscular junction and thus depress frequency-response curves. The possibility that adenosine does have a physiological role in modulating noradrenergic transmission in the rat vas deferens at low frequencies of stimulation has not been excluded in the present study.

With respect to this possibility, responses of rat vas deferens to low frequency stimulation are more sensitive to inhibition by adenosine than are responses to high frequency stimulation. Alternatively, since adenosine-mediated relaxation of coronary smooth muscle appears to be of physiological significance under anoxic conditions (cf. Berne, 1963), this may also be the case in the vas deferens. Therefore, the possibility that sufficient quantities of adenosine are released under anoxic conditions in vas deferens to inhibit noradrenaline release also remains to be investigated.

Other investigators have also utilized inhibitors of adenosine uptake and deamination when studying presynaptic adenosine-mediated feedback on noradrenergic transmission. Enero and Saidman (1977) found that dilazep and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), inhibitors of adenosine uptake and deamination respectively, reduced the mechanical activity and nerve stimulation-induced overflow of [^{3}H]-noradrenaline from the rat portal vein. These authors suggested that this resulted from raising the effective concentration of adenosine released by nerve stimulation, and that adenosine plays a physiological role in this tissue. Further support for this concept would result from blocking the effect of the inhibitors of adenosine uptake and deamination with an adenosine-receptor antagonist. However, to date, this is the best evidence that adenosine modulates noradrenergic transmission physiologically.

FUTURE WORK

Presynaptic adenosine-mediated inhibition of noradrenergic transmission is a relatively new concept. As such, many avenues of research concerning this phenomenon remain to be explored. Discussion will therefore be limited exclusively to future work in the field stemming from the present study.

The major observation in the present study is that uptake and deamination of adenosine in the rat vas deferens mask its true potency to the extent that relative potencies and dose-response relationships are meaningless unless these processes are controlled. This result could be supported by similar experiments utilizing other inhibitors of adenosine uptake and deamination, i.e., dipyridamole and EHNA respectively. Further, the present study utilized various drugs as tools assuming that the drug mechanism of action was the same as that found in other systems. More direct experimentation utilizing radioactive adenosine and radioactive adenosine derivatives would substantiate the conclusions drawn in this study.

Having established more directly the importance of adenosine uptake and deamination, similar experiments could be done in other tissues in which these processes may be more or less important with respect to the potency of adenosine and related compounds. One can predict from the present investigation that inhibitors of adenosine uptake and deamination should potentiate purine riboside potency in tissues where these processes exist.

From the present study, inactivation mechanisms appear to cause the difference between potencies of adenosine and an apparently more potent adenosine derivative, 2-chloroadenosine. It is interesting to

speculate that an adenosine related compound may be less potent than adenosine because uptake and/or deamination are more limiting to the potency of the former compound than to adenosine potency. This possibility can be tested experimentally. Should this, in fact, be the case, the conclusion that uptake and deamination should be controlled during structure-activity relationship studies of adenosine would be further supported.

The initial observation that theophylline antagonist activity appears to be potentiated by inhibitors of adenosine uptake and deamination also lends itself to further investigation. Some questions that should be asked are as follows: What is the mechanism of the potentiation of theophylline-mediated antagonism by these agents? Some possible mechanisms have already been discussed. Are other purinergic-receptors antagonists, i.e., caffeine, quinidine, etc., also potentiated by inhibitors of adenosine uptake and deamination? If in fact inhibitors of adenosine uptake and deamination potentiate adenosine-antagonist effectiveness specifically then perhaps these processes should also be eliminated in experiments which classify adenosine-receptors using adenosine antagonists.

Finally, the physiological significance of adenosine-mediated inhibition of noradrenergic transmission remains to be proven. In the rat vas deferens, experiments with inhibitors of adenosine uptake and deamination have failed to demonstrate such a physiological role for adenosine. However, the possibility that adenosine-mediated inhibition of nerve stimulation-induced noradrenaline release in vas deferens becomes significant under anoxic conditions or at low frequencies of stimulation remains to be studied. Having established a physiological

significance for adenosine in this respect in the rat vas deferens which is devoid of purinergic nerves, the source of purine riboside remains to be elucidated. As discussed previously, adenosine and/or related compound(s) may also be released from noradrenergic or cholinergic nerves or from postsynaptic neuroeffector sites.

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